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by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled HERPESVIRUS IMMUNITY IN CERVICAL CARCINOMA submitted by JOHN JACOB LYANGA in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Immunology).

DEDICATION

This work is dedicated to my wife Cornelia-Scholastica-Namageni for her understanding and encouragement and our two daughters Esther-Jacqueline-Kyoga and Stephanie-Noella-Mahulisya for their patience.

ABSTRACT

Lymphocyte transformation and antibody dependent cell-mediated cytotoxicity (ADCMC) ^{51}Cr release assays were used to detect cell-mediated and humoral immunity to HSV-2 in lymphocytes and sera from patients with cervical carcinoma. There was a high prevalence of both CMI and humoral immunity to HSV-2 in the untreated patients, but CMI alone was a more frequent finding in the treated group.

CMI was detected in 6 of 8 patients with untreated carcinoma-in-situ; 9 of 12 patients with treated cervical carcinoma; 3 of 11 patients with carcinoma of the uterine body and or ovaries, 6 of 8 healthy individuals, 3 of 5 patients with lung cancer and 8 of 18 patients with medical illnesses. Serum reactivity in ADCMC was detected in 7 of 9 patients with untreated carcinoma-in-situ; 3 of 8 patients with treated carcinoma-in-situ; 7 of 25 patients with treated squamous cell carcinoma of the cervix; 14 of 40 patients with carcinoma of uterine body and or ovaries; 2 of 6 healthy individuals and 8 of 20 patients with medical illnesses.

The presence of ADCMC reactivity in some patients with carcinoma-in-situ and squamous cell carcinoma in the presence of CMI to HSV-2 may be an indicator of the presence of a lesion containing viral structural proteins and while ADCMC serum reactivity declines after good response to therapy, demonstrable CMI persists.

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I INTRODUCTION

At the beginning of this century it was shown that antibodies carry out important protective functions sometimes in conjunction with the complement system; for example: neutralization of viruses, lysis of certain microorganisms, or their opsonization for more efficient phagocytosis. In 1921 Zinsser was the first to note that tuberculosis was accompanied by an unusual form of immunity. This seemed to be a defensive reaction but its precise nature and role remained obscure for many years. It was not possible to transfer this type of immune response by the transfusion of antibody containing serum and this prompted the speculation that there might be another form of immunity, with a separate mechanism.

Essentially two classes of mononuclear cells, lymphocytes and macrophages are responsible for the development of an immune response. The lymphocytes have a unique property of specificity and macrophages act as accessory cells generally with a non antigen-specific role. It is possible that in some reactions macrophages acquire specificity but only as a result of interaction with a lymphocyte product. The list of lymphocyte subpopulations is growing all the time but the most notable advance has been the demonstration that there are two types of lymphocytes; B and T which differ in their ontogenetic development, properties, functions and their distribution in peripheral lymphoid organs (1). Morphologically B and T cells are small

lymphocytes not distinguishable under the light microscope. The initial reports of examinations by scanning electron microsopy suggested that T lymphocytes or T-cells generally possess fewer microvilli and are smoother than the more villous B lymphocytes, but villous T lymphocytes and smoother B lymphocytes have been identified (2). T lymphocytes are thymus-dependent and arise from stem cells in the bone marrow which mature under the influence of the thymus and respond to specific antigenic stimulation but do not secrete antibody. T lymphocytes are involved in the generation of cell-mediated immunity (3,4). They participate in the rejection of tumors and allografts, delayed-type hypersensitivity reactions, activation of macrophages to resist infections, and they co-operate with the precursors of antibody forming cells. B lymphocytes are bone-marrow derived and thymus-independent. Cells derived from them synthesize and secrete antibodies which contribute to humoral immunity. B lymphocytes have surface immunoglobulin or immunoglobulin like receptors and these constitute the recognition units of the reactive cells (5). The nature and number of functional receptors on T cells is still a matter of considerable controversy (6). Even more recently a subpopulation of K cells has been identified but it is not yet fully known how these cells develop. Most investigators however agree that K cells are effector cells in many systems of antibody-dependent cell-mediated cytotoxicity (7).

The purpose of this study was to assess the immune response to herpes simplex virus type 2 in patients with either Carcinoma of the Cervix in situ or invasive squamous cell carcinoma of the cervix.

II SURVEY OF LITERATURE

(A) Cell-Mediated Immunity (CMI)

(1) Nature of Cell-Mediated Immunity

A simplified definition of cell--mediated immunity is that it is an immunological response manifested by some interaction between antigen and immunocompetent lymphocytes with a possible intermediate step consisting in the processing or presentation of antigen by macrophages (8).

T cells involved in CMI are antigen sensitive and their response is possibly also influenced by B cells or antibodies. Certain special conditions must be met before antigens can induce T cell response; for example it is known that live intracellular parasites evoke delayed type hypersensitivity while killed vaccines or antigen extracts containing some of the same antigenic determinants as live organisms evoke CMI only if incorporated in Freund-type complete adjuvants. Ichiki and Parish in 1972 (9) showed that Salmonella adelaide flagellin which was enzymatically degraded could induce CMI whereas unmodified antigens induce only an antibody response. Salvin in 1958 (10) demonstrated that when minute doses of diphtheria toxoid or of ovalbumin were complexed with antibody in appropriate proportions, they could evoke cell-mediated immune responses.

CMI responses are found in some forms of contact sensitivity as well as in bacterial, viral, fungal and parasitic infections, homograft immunity, tumour immunity and certain autoimmune diseases such as experimental allergic encephalomyelitis (11). There is ample evidence that cell-mediated immune responses are effected by specifically sensitized T lymphoid cells (12,13).

(2) Delayed Hypersensitivity

The term "specific cell-mediated immunity" has been introduced to cover those immunological conditions in which the immune activity is performed by circulating lymphocytes these include the allergic skin responses known generically as "delayed hypersensitivity". This term "delayed hypersensitivity" has been used for many years for a group of allergic reactions, especially in the skin, which, once the individual is sensitized, take 24 - 48 hours to develop on subsequent contact with the specific antigen. This period of development is in marked contrast to that of reactions mediated by humoral antibody which occur between 15 and 30 minutes after contact with antigen in the case of anaphylactic reactions or between 4 and 8 hours in the case of Arthus reactions (14,15). The notion that cells can effect immune responses directly without antibody as intermediary, slowly gained acceptance as a

manifestation of general import in immunity. The progress in this field was slow and limited because the only type of response through which cell-mediated immunity could be studied was the manifestation of delayed hypersensitivity. It was not until in the early 1940's that investigators Landsteiner and Chase (16,17) and Chase (18) showed that contact hypersensitivity induced in guinea pigs to simple chemicals like picryl chloride, 2, 4 - dinitrofluorobenzene or killed tubercle bacilli suspended in paraffin oil could be transferred to normal guinea pigs by cell transfer. The transfer was performed by injecting inbred male albino guinea pigs intravenously or intraperitoneally with living peritoneal exudate cells obtained from sensitized animals. It was not however, possible to transfer such hypersensitivity by using even very potent antisera, or heat-killed exudate cells. It is now clear that besides peritoneal exudate cells, buffy-coat cells from blood, thoracic duct cells, spleen cells and lymph node cells are effective in transfer.

Chase recognized that such contact hypersensitivity resembled in kind the long known bacterial allergies - represented typically by the tuberculin reaction - that had been distinguished from antibody mediated skin reactions of immediate (anaphylactic) hypersensitivity. Chase described both these slowly developing but specific forms of skin reaction to antigen as "delayed

hypersensitivity".

From the work of Landsteiner and Chase, and Freund (19,20,21) it was confirmed that dead tubercle bacilli produced much higher levels of sensitization if incorporated in oil; and they used paraffin oil mixed with vaseline. This led them to produce delayed hypersensitivity to specific antigens by incorporating them in an adjuvant of killed mycobacteria in oil, subsequently known as Freund's complete adjuvant.

(3) Histopathology of Delayed Hypersensitivity Reactions

The impetus to study the histopathology of delayed hypersensitivity in more detail was prompted by the work of Waksman and Matoltsy in 1958 (22) when they observed that the effect of antigen on sensitive mononuclear cells in tissue culture might be stimulating rather than cytotoxic. Further there was evidence in experimental auto-allergies that the "perivascular island" reaction described by Gell in 1959 (23) was accompanied by a histiocytic invasion of the antigen-containing parenchyma, which was apparently destroyed by these invading cells.

Histological studies show (24) that in hypersensitivity skin lesions that develop at the site of antigen application or injection, the essential change is local tissue damage in the neighbourhood of an

infiltrate of mononuclear cells. This is an immunologically specific phase of the reaction and takes place during the first 12 hrs or so; and subsequent histological changes are those of a secondary inflammatory response of a non-specific character. The mononuclear cells of the initial infiltrate have been morphologically identified as lymphocytes, macrophages in various proportions.

In 1962 (25) Waksman classified the lesions as: (i) The invasive-destructive lesion where lymphoid cells are associated with focal areas of destruction of antigen containing parenchyma. This type of lesion is often seen in experimental animal studies in the rejection of tumour or skin; and graft-versus-host reactions; as well as the lesions of auto-immunity; and (ii) Vasculonecrotic areas, as seen in tuberculin reactions where fibrinoid or necrotic changes in blood vessel walls and adjacent parenchyma are associated with perivascular infiltrates of mononuclear and polymorphonuclear leucocytes. It seems that the principal events of delayed hypersensitivity reactions are local perivascular accumulation of sensitive cells and there is an invasive-destructive process directed at antigen-containing elements.

(4) <u>Effector Cells of Cell-Mediated Immunity</u>

Experiments of Arnason et al in 1962 (26) and

Jankovic et al 1962 (27) in which rats were thymectomised at birth or during the first week of life showed that Arthus reactivity as well as delayed-type hypersensitivity to bovine serum albumin (BSA) were suppressed. When these rats were tested as adults they failed to develop auto-allergic encephalomyelitis, and there was a significant depression of tuberculin sensitivity. Immunological dissociation was apparent in these experiments as natural hetero-agglutinin titers remained unchanged and there was no abnormal suppression of serum gamma globulins. Bursectomy done in 2 days old chickens has no effect on skin allograft rejection nor does it impair delayed-type hypersensitivity to spinal cord lipid antigen or tuberculin (28,29). These findings are in complete agreement with the current understanding that T cells are thymus-derived and are mediators of cell-mediated immunity whereas B cells migrate in chickens from the bursa of Fabricius, and in mammals the bone marrow, and are mediators of humoral immunity. When thymectomy is done at birth, before lymphoid peripheralization has taken place, cell-mediated immune responses cannot be evoked in later life and B cells have no appreciable effect-on this type of immunity. The chicken is an ideal model to study the separate functions of B and T cells while it is not so easy in mammals where the bursa-equivalent tissue is yet to be identified.

The radio-autographic and election microscope studies by Gowans and Knight in 1964 (30) and Marchesi and Gowans in 1964 (31) on the route of re-circulation and migration of labelled lymphocytes in rats was fundamental to further work on tracing the fate or peripheralization of lymphoid cells and thymocytes. Markers of thymocytes by Joel et al in 1972 (32), Davies in 1969 (33) and Miller & Sprent in 1971 (34) demonstrated that they migrated to thymus-dependent areas of secondary lymphoid tissue - to the periarteriolar sheath of spleen and paracortical area of lymph nodes. Antilymphocyte serum (ALS) treatment (35) profoundly suppressed cell-mediated responses. It was also shown that proliferation of T cells occurs in thymus-dependent areas of lymph nodes and spleen. Stimuli that do not provoke a response in these same areas are not associated with CMI. There is evidence to indicate that antisera against T lymphocyte markers will eliminate specific effector cells from populations of immune lymphocytes (36,37).

It has been shown that dividing cells appear in the thymus-dependent area 1-2 days after intravenous injection of dead bacteria. The peak of response depends on the type of antigenic stimulation and is dose dependent; for example it is 6 days for Listeria in the mouse (38) and occurs later to stimulation with tubercle bacilli (39). The dividing cells are large blasts with

pyroninophilic cytoplasm initially but later on become pyroninophilic small lymphocytes.

(B) In Vitro Assessment of Immunity

(1) Lymphokines

In 1969 Bloom & Bennet; David; and Dumonde et al (40,41,42) observed that it was possible to define specific cellular responses to antigens by in vitro production of soluble factors collectively referred to as lymphokines. T lymphocytes appear to perform the activities of CMI by releasing these factors. However, certain nonspecific stimulators of T lymphocytes for example the plant mitogen phytohaemagglutin (PHA) can also cause release of some of these factors or mediators.

Lawrence's transfer factor (TF) can transfer in man delayed cutaneous hypersensitivity to tuberculin, streptococcal proteins and other antigens (43,44).TF has been used prophylactically or therapeutically in Wiskott Aldrich Syndrome (45,46,47) and is currently undergoing clinical trials in disorders associated with defective cell-mediated immunity.

Migration inhibition factor (MIF) has been extensively studied by Rich & Lewis in 1943 (48); George and Vaughan, 1962 (49) and David et al in 1964 (50). It has been found that in guinea pigs the degree of

macrophage inhibition correlates very well with delayed skin reactivity of cell donors.

Many other factors have been described and these include chemotactic factors for monocytes, eosinophils and neutrophils (51,52), colony inhibition factor (53), interferon (54), blastogenic factor or mitogenic factor (55), lymphotoxins (56), macrophage aggregating factor (57), proliferation inhibition factor and skin reactive factor (58). Bloom and Glade in 1971 compiled tables of lymphokines (59) (Table 1).

Evidence is now overwhelming that lymphokines may act on lymphocytes, macrophages, fibroblasts and parenchymal tissue cells and this is the basis for their participation in the peripheral expression of cell-mediated immunity. They are thus involved in activation and recruitment of inflammatory cells in delayed hypersensitivity (60,61), allograft rejection (62), and graft-versus-host reactions (63) and in parenchymal tissues damage accompaning some forms of autoimmunity (64). Although at the present level of purification it is certain that these factors are non-antibody mediators of cellular immunity, purer forms must be prepared and critically tested before their individual definitive roles in CMI can be clearly defined.

(2) Lymphocyte transformation in culture

Lymphocytes found in human peripheral blood are essentially nondividing cells; and some can remain in this state in the body for many years while still retaining the potential to proliferate or transform when exposed to appropriate stimuli (65,66). When these cells are cultured alone in medium, they remain dormant and the RNA and DNA synthesis is so minimal that it cannot be quantitated.

In 1960 Nowell (67) observed that a red bean extract, Phaselous vulgaris, phytohemagglutinin, (PHA), stimulated most human peripheral blood lymphocytes. In fact, PHA had been used since 1908 in removing human red cells from leukocyte suspension by agglutination. The stimulation or activation of lymphocytes forms the basis of lymphocyte transformation tests. Morphologic alterations and measurements of RNA, DNA (68) and protein synthesis using radiolabelled precursors have all been used as indicators of the response. Although isotopic incorporation is now the most sensitive and easily quantitated method, because of our imcomplete knowledge of the biochemistry of lymphocyte transformation, it is not clear which of these changes is more pertinent to transformation.

A large number of unrelated agents which can stimulate human lyphocytes independently of prior

exposure and apparently without any immunologic specificity are frequently referred to as nonspecific mitogens. These include phytohemagglutinin, Concanavilin A (Con A), from jack bean, Canavalia ensiformis and Pokeweed mitogen, (PWM), from pokeweed, Photolacca americana (69). Pure forms of these mitogens are available and they are among the most potent lymphocyte stimulators; and can activate up to 90% of normal human peripheral blood lymphocytes in culture. Some of the other nonspecific lymphocyte activators include divalent cations of mercury, zinc and nickel (70,71,72); and bacterial products for example streptolysin S (73). PHA and CON A stimulate T cells while PWM stimulates both T and B although some PWM fractions are pure T cell stimuli. Cell specificity of various mitogens (Table 2) has been demonstrated (74).

The observation made by Pearmin et al in 1963 (75) that PPD stimulated a small proportion of lymphocytes to transform in vitro only if the leukocytes were from donors with delayed cutaneous reactions to PPD, was most important. Lymphocytes from donors with negative skin tests failed to react to PPD in vitro. The proliferative responses was demonstrated to have hapten-carrier specificity in that little or no proliferative reaction was obtained in response to other proteins, the protein carrier itself or the hapten by itself.

There appear to be distinct receptors for mitogens

on lymphocytes while those for antigens can be separately defined. Lymphocyte cultures are generally examined for cell proliferation after 3 to 7 days and usually the peak transformation is on day 3 for the mitogen and day 6 for the antigens. Sensitive lymphocytes which have been thoroughly purified to exclude adherent cells respond very poorly to stimulation by the sensitizing antigen in mixed leucocyte culture. The response however can be significantly increased by addition of autologous macrophages (76).

Before the classification of B and T cells considerable attempts were made to correlate the immune state of lymphocyte donor in regards to a specific antigen and the in vitro lymphocyte transformation response. In 1966 Mills (77) showed that in vitro lymphocyte transformation by antigen could be corelated with the in vivo state of cellular hypersensitivity in the host; and that it could be demonstrated earlier than in vivo cellular hypersensitivity. Girard et al (68) found, too, that lymphocyte transformation can be used to detect antibody producing cells because the response correlated well with antibody production. However, Smith studied rubella virus in 1973 (78) and found that lymphocyte transformation by rubella virus could be detected in sensitised individuals even in the absence of detectable in vivo antibody titers and lymphocyte

transformation in this instance was more sensitive in detecting prior rubella virus exposure.

Although generally speaking, lymphocyte transformation response reflects T cell function, depending on the nature of the antigen, it can reflect cells mediating cellular or humoral immunity or both. Whatever the response reflects, it can be safely concluded that antigen-induced transformation demonstrates that there has been prior immunologic experience with that particular antigen whether it be bacterial, viral, fungal, protozoal or plant material.

The interpretation of lymphocyte transformation results must always be approached with caution. It should be noted that variations in the normal persons can be wide; and among factors which can influence the results are age, immunosuppressive drugs, irradiation, circadian rhythm of T cell activity, doses of mitogens and antigens used, and duration of cultures (79,80,81). It is therefore essential that optimal conditions are pre-determined and all the essential controls included in the tests.

(3) Antibody-dependent Cell-Mediated Cytotoxicity or (ADCMC)

In 1965 Moller (82) described a system in which antibody-coated target cell lysis in vitro could be

mediated by antisera raised against the targets, in the absence of complement, but effected by non-sensitized lymphoid cells. She stressed that there must be close contact between lymphoid cells and targets. The studies were done using rabbits and rats with Sarcoma. This system has been extensively studied and in descriptive terms, the cells which are destroyed by addition of lymphoid cells are called target cells or targets and the cells which achieve this are called effector or killer cells.

The work of Moller has been firmly established by Perlmann and Holm (83) and Maclennan (84). The target cells are radiolabelled with chromium - 51 (^{51}Cr), and target cell lysis can be measured by specific release of ^{51}Cr (85,86). Kovithavongs et al in 1974 (87,88) termed the phenomenon of this system Antibody-Mediated cell-dependent immune lympholysis (ABCIL) because the target cells used were lymphocytes. Studies were done on immunity to tissue sensitization to detect histocompatibility antigen sensitization after pregnancy, in multitransfused patients, patients on chronic hemodialysis or after organ transplantation. Although the biological significance of ABCIL remains to be determined and its use routinely applied in the field of transplantation, it has been shown that the system is sensitive and detects specific sensitization which is wide and extends beyond the present restrictions of the

serologically defined components of the HLA system.

The phenomenon of target cell lysis in this system is a distinctive and specific immunologic process and quite separate from complement - dependent cytotoxicity. Maclennan (86) suggested that it was effected by what he called "Cytotoxic B cells." However, other workers (89) have called the effectors "K" or killer cells, and most investigators are of this latter view. It has been shown that K cells or effector cells in this system recognize and bind onto target by virtue of surface receptors for Fc region of an IgG antibody (90). Binding alone is not a sufficient condition for cytotoxicity because targets coated with C3b (but not IgG) are not killed even though the effector cells have receptors for this complement component (85,91). The ontogeny of the effector cells has not been clarified.

(C) Herpes Simplex Viruses and Cervical Carcinoma

(1) Recurrent Herpes Simplex virus infection

Most of the population has been infected by herpes simplex virus by the time of adolescence. Herpes simplex virus may cause encephalitis, generalized infection of the newborn, which can be fatal, and herpes simplex virus type 2 (HSV-2) cause genital lesions and has been linked with cervical carcinoma. Herpes Simplex type 1 (HSV-1) causes lesions traditionally deemed to be above the waist - predominantly in the form of circum-oral

(herpes labialis) disease. However, the commonest sequel to herpes simplex virus infection, and indeed to all herpes viruses (Epstein-Barr virus - EBV, cytomegalovirus, - CMV, and varicella/zoster virus) is viral persistence and, except for EBV, the possibility of troublesome and sometimes painful-recurrent disease (92). Patients with a clear clinical history or serological evidence of previous HSV - 1 or HSV - 2 primary infection exhibit recurrent infections caused by the same virus and localized to specific areas, for example, the lips, cornea or genitalia. This observation weakens the contention that exogenous reinfection generally occurs although it has been demonstrated in mice and Cebus monkeys (93) that exogeous genital reinfection is possible (Table 3).

Recurrence of herpesvirus infections may be subclinical or be clinically obvious while the term recrudescence is preferably restricted to the clinically manifest infection. During latent periods, there is usually no demonstrable evidence of these viruses either in the host tissues or in the secretions. This has perplexed virologists for many years but there is now evidence to indicate that special techniques can be used to demonstrate these viruses even during latent periods. Gerber and Lucas (94) showed that the EBV reappeared if the lymphocytes of the previously exposed persons were cultured with 5-Bromodeoxyuridine; and Craig and Nahmias

isolated HSV-2 from peripheral buffy coat blood cells in patients with meningitis (95). HSV-1 has been isolated from trigeminal ganglia removed at autopsy (96,97). Studies have also been done to show that the virus may persist in the sacrosciatic spinal ganglions of mice after inoculation in the footpads (98,99). The virus could be demonstrated by coculturing ganglions with susceptible cells but not if the susceptable cells were cultured with homogenized ganglion cells. This suggests that the virus is in a non-replicative form or may persist in an eclipsed form (100).

(2) Immunity in Herpes Simplex Virus Type 1 and 2

Andrewes and Carmichael (101) were the first people to comment on the inconsistency that antibodies to HSV-1 can be demonstrated in both groups of patients, those susceptible and those not susceptable to infections. This has been confirmed by a number of investigators in the field and associations of HSV infections and both humoral and CMI demonstrated. Russell in 1973 (102) studied volunteers with frequent and occasional recurrent cold sores by using tests of lymphocyte transformation - HSV-1 antigen, and complement fixing antibodies. It was shown that those persons who had recurrent infections had detectable antibodies and CMI to HSV-1 and there was no demonstrable immune response in those without infection. It has been suggested that

these patients have another form of immunity other than the conventional humoral and CMI; and specifically the role of macrophages has been postulated (103).

Further work on patients with cold sores by Russell et al (104) has produced evidence of the presence of specific immunity to herpesvirus antigen in tests of lymphocyte toxicity, ADCMC and skin tests. It has been argued that CMI evident in these patients may not be important in effecting resistance to recrudescent HSV-1 infections; and that its main role may be in localizing herpes lesions (105). It is known that the incidence of herpes zoster infections is increased in patients with diseases or who are on drugs associated with depressed CMI (106,107,108). In fact, persons with profoundly depressed CMI tend to develop unusually persistent and/or disseminated HSV- infections rather than recurrent cold sores (109,110).

A positive leukocyte migration inhibition (LMI)test was demonstrated in 62% of 24 persons susceptible to recurrent herpes labialis but in none of 10 persons without such infection (111). Although the LMI test was less sensitive than the lymphocyte transformation test using HSV-antigen, the two tests correlated well; providing additional evidence that CMI in these patients is not defective.

(3) Cross-reactivity in Herpes Simplex Viruses

The question of antigenic heterogeneity of herpes simplex virus strains has been investigated extensively since the isolation of the virus by Gruter in 1912. While early reports suggested that all strains were homogenous, more recent evidence indicates that antigenic differences and similarities among herpes simplex virus strains do exist, but the degree is open to question. Further it is not entirely clear in most cases which component of the herpesvirion is responsible for the relatedness between herpes simplex viruses and several other herpesviruses for example varicella zoster or Simian B virus. It can be inferred that in the cases in which cross-neutralization occurs, for example between HSV-1 and Simian B virus, antigens on the virus envelope are involved.

Relatedness between HSV-1 and HSV-2 is demonstrated by presence of cross-reactivity of serum specimens of persons who have antibodies to both types of HSV. It can be shown that antiserum prepared against HSV-1 neutralized HSV-2 and vice versa. The specificity of the reaction, defined as the ratio of antiserum titers against homologous and heterologous viruses may vary from person to person (112).

Plummer in 1964 (113) and Wheeler et al 1969 (114) used kinetic neutralization techniques in an attempt to

[illegible]

[illegible]

[illegible]

[illegible]

differentiate the subtypes of HSV. In the kinetic neutralization tests virus inactivation curves can be prepared by plotting % survival of virus against time after serial dilutions of antisera have been added at time zero. The time for neutralization of 50% of virus was determined. In order to compare the required time for 50% neutralization of homologous virus with time required for 50% neutralization of heterologous virus, normalized 50% neutralization times (NT) were calculated as ratios:

$$\frac{\text{Time for neutralization of 50\% of heterologous virus}}{\text{Time for neutralization of 50\% of homologous virus}} \times 100$$

for heterologous virus, it would be greater than 100; and the cross-reactivity of HSV-1 and 2 could be shown.

There have been a number of other techniques used in distinguishing antibodies to HSV-1 from antibodies to HSV-2 for example complement fixation tests (115) and indirect cell membrane immunoflorescence (116).

The concept of using ratios of titers or times of reactions has continued to be applied. Smith et al in 1972 (117) studied the patterns of antibody response in humans to herpesvirus type 1 and 2 using ^{51}Cr release assay. They used the Index;

$$\frac{\text{Log10 Antibody to HSV-2} \times 100}{\text{Log10 Antibody to HSV-1}}, \text{ or II/1,}$$

to indicate specificity of the antibodies. He

demonstrated that all sera with an index of 80 or less had all reactivity removed by absorption with hamster monolayer cells infected with HSV-1; and all sera with an index of 101 or greater had residual activity.

Adam and his coworkers (118) did seroepidemiologic studies on HSV-1 and HSV-2 in patients with carcinoma-in-situ and they used the index II/1 at threshold 85 and 95. They found that by using X^2 the groups of patients with carcinoma-in-situ and control were maximally distinguished showing a significantly higher number of patients with carcinoma-in-situ with HSV-2 antibodies than the control group of patients when threshold was taken as 95.

Most investigators now agree that type specific HSV antibodies can best be determined by the use of ratios and when they are read in isolation the interpretations can be misleading. Herpes simplex viruses (HSV) have important differences (Table 3).

(4) Immunity to HSV-Summarized

From the evidence available it is clear that some form of immunity develops following herpesvirus infection although its role is not entirely clear. Studies do indicate that cell-mediated immunity probably plays a role in limiting herpetic lesions during reinfection or recurrences and that humoral immunity may

be one of the factors which maintain the virus in the latent form. Persistent and/or disseminated forms of cutaneous herpesvirus infection occur among patients with severely compromised CMI for example in immune deficiencies.

Although HSV-1 and HSV-2 are well-defined subgroups of herpesvirus, there is extensive cross-reactivity demonstrated by serologic techniques which detect both anti-HSV-1 and anti-HSV-2 antibodies, and the problem is even more complex in a patient with both types of infection. There is thus strong antigenic relationship between these viruses and indeed they may share some aspects of immunity.

It is not easy to interpret titers of anti-HSV-1 and anti-HSV-2 accurately when read in isolation and that is why the use of ratios of the titers is being applied more often. Similar indices have been adopted when lymphocyte transformation tests are performed in humans or animals in an attempt to differentiate between HSV-1 and HSV-2 infections.

(5) Associations of HSV-2 with Cervical Carcinoma

The transformation of cells by virus is associated with the permanent transfer of virus genetic material to a cell. A number of animal viruses including certain members of the herpes group have the ability to

transform mammalian cells with loss of contact inhibition, and some of these cells change into malignant phenotype. In 1972 Rapp (119) was able to transform hamster embryo fibroblasts when cultured with HSV-2 previously exposed to ultraviolet radiation. Cell lines established from several clonal isolates of transformed foci induced fibrosarcoma when injected into newborn hamsters. The tumors were invasive with pulmonary metastatic lesions. Herpesvirus specific antigen was presenting 1-5% of the transformed cells contained in the cytoplasm and the tumour bearing hamsters developed detectable HSV-2 neutralizing antibodies.

There is evidence to support the etiological role of herpesviruses in a lymphoma of chickens (Marek's disease), renal adenocarcinoma in Rana pipiens (frogs) and a lymphoma leukaemia of non human primates, rabbits and guinea pigs (120). In humans EBV is associated with Burkitt's lymphoma, nasopharyngeal carcinoma and possibly Hodgkin's disease. In the mid 1960's Martin suggested marital and coital factors may have a causal relationship with cervical carcinoma (121), and currently HSV-2 is associated primarily on an epidemiological basis with cervical carcinoma. There is also a suggested relationship between HSV-1 infection and carcinoma of the lip.

Zanda et al in 1973 (122) determined the incidence

of antibodies to herpes simplex virus type 1 and 2 in females with cervical atypia and different stages of cervical cancer. It was found that although some subjects from all groups had anti-bodies to HSV-1 and HSV-2 the percentage of patients with anti-HSV-2 antibodies was significantly increased in those groups with cervical atypia (50%), cervical carcinoma-in-situ (48%), and invasive carcinoma (50%). The overall incidence of anti-HSV-2 among the control group was 19%. Patients with invasive carcinoma had the highest geometric mean titers for both HSV-1 and HVS-2.

McDonald and his colleagues (123) studied neutralizing antibodies to HSV-1 and HSV-2 in 57 women with invasive carcinoma of the cervix, 50 with carcinoma of the cervix-in-situ and 37 with cervical dysplasia. The ratio of type 2: type 1 herpesvirus neutralizing antibody was calculated and sera in which the ratio was equal to or greater than 0.85 was called type 2. The results were described as inconclusive.

A herpesvirus type 2 induced tumor specific antigen in cervical carcinoma was reported by Aurelian et al (124). A micro-quantitative complement fixation test was used to detect antibody to tumor specific herpesvirus type 2 induced antigen (AG-4) in sera from patients with cervical carcinoma. The antibody was detected in 35% of 20 patients with atypia, 73% of 32 patients with carcinoma-in-situ and 89% of 27 patients with untreated

invasive carcinoma. However, there was almost no anti-AG-4 in the control group of women without cancer matched for age, race and socioeconomic class. Further, antibody to AG-4 was absent in 23 patients tested after successful therapeutic-radiation or hysterectomy, and was detectable in 3 patients with recurrent carcinoma after therapy. The reactivity of AG-4 could be demonstrated in 5 out of 6 squamous cervical carcinoma biopsies while none could be demonstrated in a normal cervical biopsy and one with adenocarcinoma. The data suggested that AG-4 may be of diagnostic and prognostic significance and an active role of HSV-2 in cervical carcinoma.

Kawana et al 1976 (125) studied antibodies to AG-4 antigen in Japanese women with cervical carcinoma and found that the prevalence of this antibody was only 47% as compared to 7% in the controls but Aurelian's et al studies had shown it to be more frequent in patient with cervical carcinoma (vide supra). Kawana et al considered the possibility that HSV-1 could be one of the factors in the etiology of cervical carcinoma.

Goldstein and his coworkers (126) studied 14 patients with proven invasive carcinoma of the cervix and one with adenocarcinoma and 7 control patients with benign uterine tumors by inhibition of leukocyte migration when peripheral leukocytes were incubated with autologous tumor. When autologous squamous carcinoma

cells in lymph nodes or cervix were incubated with leukocytes, inhibition of migration could be demonstrated but not when lymph node cells and cervix were from the control group.

Overall, epidemiologocal studies indicate that the cervix is affected more often than the external genitalia and that epidemiological patterns of HSV-2 infection and cervical carcinoma are similar. After the primary infection it takes at least 6 years before carcinoma-in-situ develops.

Table 4 is an outline of the associations of herpesvirus and cancer (127).

Table 1

Stimulated lymphocytes elaborate lymphokines with these biological activities.*

Factor	Activity
1. Transfer factor (TF)	Transfers CMI in vivo. sensitizes uncommitted T lymphocytes
2. Mitogenic or Blastogenic factor (MF or BF)	Induces blastogenesis & cell division of normal lymphocytes in vitro.
3. Migration inhibition factor (MIF)	Inhibits migration of macrophages in vitro.
4. Macrophage Aggregation factor (MAF)	Agglutinates suspended macrophages in vitro. Attracts macrophages to site of infection.
5. Chemotactic factor (CF)	Migration of granulocytes through micropore filter. Attracts macrophage to site of infection.
6. Lymphotoxin (LT)	Cytotoxic to cells in culture in fibroblasts destroys pathogens and complement-independent.
7. Skin reactive factor (SRF)	Monocytic skin reaction, delayed type hypersensitivity like lesions in normal guinea pig (histology).
8. Inteferon	Protects against viruses, inhibits viral replication.

*Wld. Hlth. Org. Rep. Ser. (1973) (8), modified.

Table 2

Cell Specificity of various mitogens*

Mitogen	Type of Lymphocyte activated	Comments
PHA	T	will stimulate
CON A	T	purified T cells. but is macrophage-dependent
Sodium periodate	T	May stimulate B but only in presence of T cells.
Endotoxin	B	May activate some T cells but not to proliferate.
Anti-immunoglobulin	B	
Polynucleotides	B	
Other macromolecules, KLH, DNP-KLH, DNP-BSA, dextran sulfate	B	
Trypsin	B	
PWM	T and B	some PWM fractions are pure T Stimuli.
ALS	T and B	cell specificity varies with
preparation		
Zinc	unknown	
Mercury	unknown	
Walnut extract	unknown	
Corn extract	unknown	

*Oppenheim, & Rosenstreich 1976 (74)

Abbreviations
ALS antilymphocyte serum
CON A conconavalin A
DNP-BDA 2-4 dinitrophenol bovine serum albumin
DNP-KLH 2-4 dinitrophenol keyhole limpet hemocyanin
KLH Keyhole limpet hemocyanin
PHA phytohemaglutinin
PWM pokeweed mitogen

Table 3

Differences between Herpes-Simplex Viruses (HSV)*

Type 1 and 2.

CHARACTERISTIC:	HSV-1:	HSV-2:
1. Clinical	Infects primarily non-genital sites.	Infects primarily genital sites
2. Epidemiologic	Transmission primarily via nongenital	Transmission primarily via genital route (venereal or mother-to-newborn.)
3. Biochemical		
DNA guanine Cytosine (Mols %	67	69
Homology of Viral DNA's	Around 50%	
OTHER: Electrophoretic mobility of several membrane Glycoproteins but not of capsid proteins		
4. Biologic		
a. Chick embryo (chorioallantioc membrane.)	small pocks	large pocks
b. Mice-genital or intramuscular inoculation	less neurotropic	more neurotropic
c. Tissue-culture-cells	Differences between 2 virus types in their ability to propagate their cytopathic characteristics or plaque size in certain cell culture lines.	

*Nahmias et al 1973 (100).

Table 4*

Summary of the data associating herpesvirus with cancer.

1. HSV-2 is transmitted by sexual contact.

2. Cervical cancer essentially behaves as a venereal disease.

3. HSV-2 is an ubiquitous human pathogen.

4. HSV-2 can cause latent infections.

5. The mean age for infection with HSV-2 precedes by six years mean age for development of pre-invasive cervical cancer.

6. Guilt association: Herpesvirus Saimiri, Lucke frog adenocarcinoma, Marek's disease virus.

7. HSV-2 and HSV-1 cause transformation of hamster cells in tissue culture, transformed cells cause tumors in hamsters and induce neutralizing antobody to HSV-2 or HSV-1 respectively.

8. Prevalence of antibody to HSV-2 is significantly higher in patients with cervical cancer than in control groups.

9. The prevalence of antibody to HSV-2 is similar in patients with atypia, carcinoma-in-situ and invasive cancer.

10. An "Early" Antigen present in cells infected with herpes simplex virus has been observed in cervical tumors.

11. Herpes structural antigens have been shown in exfoliated cervical tumor cells.

12. HSV-2 has been isolated from a tissue culture established from a carcinoma-in-situ.

13. Carcinoma of the lip may occur at the site of HSV-1 recrudescent infection.

*Aurelian (1974) (127)

Table 5

Distribution of Subjects Studied

Diagnosis	number	Age-Range	mean age ±SD
1. Carcinoma-in-situ	17	22-56	33 ± 9
2. Squamous cell Carcincma of Cervix-stage 1	14	34-74	52 ± 10
3. Squamous cell Carcincma of Cervix-stage 2	10	34-75	57 ± 13
4. Squamous cell Carcincma of Cervix-stage 3	1	57	57
5. Adenocarcincma of Cervix	5	27-65	41 ± 14
6. Carcincma of the uterine body and/or ovaries	40	33-77	54 ± 20
7. Vaginal and Vulva carcinoma	2	44-77	60 ± 23
8. Lung cancer	5	41-68	57 ± 12
9. Healthy Individuals	10	20-44	23 ± 7
10. Medical illnesses	21	20-82	50 ± 20
TOTAL	125		

III MATERIALS AND METHODS

(A) Materials

(1) Subjects Studied

Subjects were studied after giving informed consent. A total of 125 subjects were studied and these included patients who were attending a cancer clinic at the Dr W.W. Cross Cancer Institute; patients admitted to the University Hospital for cone biopsy because of suspicious cervical cytology; patients studied at random from one medical ward and healthy individuals from the nursing staff.

The age range of subjects studied was 20-82 years and all were females except 4 males who had lung cancer. All the patients had lesions documented on histopathological grounds and these included: 17 patients with carcinoma of the cervix-in-situ; 25 with invasive squamous cell carcinoma of the cervix; 5 with adenocarcincma of the cervix, 40 with carcinoma of the body of the uterus and or ovaries; 2 with vaginal and vulva carcinoma and 5 with carcinoma of the lung (Table 5). Some patients were tested before and after therapy.

(2) Mononuclear cells

Sterile reagents, tubes and glassware were used. All reagents were kept at 4°C but were allowed to warm

up to room temperature just prior to use. Ficoll-Isopaque (Ficoll, Pharmacia, Fine Chemicals, Uppsala, Sweden; & Laboratory Products Nyegaard and Co. AS. Oslo) and TC medium 199 (Gibco) are reagents used in the preparation of mononuclear cells . Ficoll-Isopaque contains a mixture of 31.2 parts of 9% solution Ficoll and 13 parts of 33.9% Isopaque (75% sodium metrizoate) and has a final specific gravity of 1.076-1.078.

Samples of peripheral venous blood were collected into heparinized vacutainer tubes (Becton, Dickinson Canada Ltd.) and gently shaken. The blood was then spun at 1200 revolutions per minute (rpm) for 10 minutes at 23°C (IEC centrifuge model PR-6 Needham Hts. Mass.) The plasma was removed and set aside. The buffy coat was pipetted off and mixed with an equal volume with Tc medium 199 containing 5% of heat inactivated fetal calf serum. The blood was spun again for the second time to maximize the yield of lymphocytes, and the two harvests were pooled. Aliquots of about 5 mls of Ficoll-Isopaque were put into tubes and an equal volume of cells and medium were gently layered onto it without disturbing the interface. The tubes were then spun at 1500 rpm for 30 minutes after which the broadened interface layer which contained mostly mononuclear cells was pipetted off. This was diluted by the addition of volumes of TC medium 199 and washed 3 times in the same. The final suspension of cells was made into TC medium 199 for

ADCMC test and in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco) for lymphocyte transformation test. The cells were counted using a hemocytometer under a microscope and appropriate cell concentrations prepared.

(3) Serum

(a) Preparation of AB Serum

Peripheral venous blood was collected from known AB blood group persons into a donor set. The blood was allowed to clot and spun at 1500 rpm for ten minutes. The serum was then pooled and stored in aliquots of 10 mls in tubes and stored at - 70°C.

(b) Serum for ADCMC

Peripheral venous blood was collected into vacutainer tubes and allowed to clot. The blood was then spun at 1500 mpm for 10 minutes. The serum was then pipetted and aliquoted in 2-3 mls in tubes.

(c) Fetal Calf Serum

Fetal calf serum was obtained from Microbiological Associates, Bethesda, Maryland in 100 ml bottles. It was heat inactivated before use.

(d) Decomplementation

All the sera were decomplemented by heating in

a waterbath at 56° for 30 minutes. After decomplementation sera and plasma were spun at 1200 rmp for 10 minutes to remove debris. The sera or plasma were then stored at - 20°C.

(4) Lymphocyte Stimulating Agents:

(a) Antigens

(i) HSV-1 antigen

The HSV-1 antigen was provided by Dr. C.P.C. Bradstreet, Director, Standards Laboratory, Central Public Health Laboratory, Colindale Avenue, London, England. The virus was grown in monkey cells (VERO) and the antigen prepared was of proven antigenicity in complement fixation tests. It was used after inactivation with 0.3% B-propiolactone, since it was found that a live virus preparation produced much less lymphocyte stimulation than inactivated antigen (111). The antigen was stored at 4°C in 5 ml bottles. A control antigen was prepared from uninfected tissue culture cells similarly treated.

(ii) HSV-2 antigen

HSV-2 antigen was prepared from a stock of HSV-2 obtained from the Provincial Laboratory, University of Alberta Hospital, Edmonton, Alberta. HSV-2 was grown in a human amnion cell

line (HAE 70) obtained from Dr. J.M.S. Dixon. The control antigen was prepared from uninfected cells. The HSV-2 antigen and control antigen were prepared in aliquots of 1 ml and stored at -20°C.

(iii) Streptokinase - Streptodornase (Varidase)

Varidase (Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y. 10965) was stocked in concentrations of 10,000 uts/ ml and stored at -20°C.

(iv) Vaccinia Virus

Vaccinia Virus (Dryvax-Wyeth, Marietta, Pa.) was used in a final concentration of 10^5pfu/ml (plaque forming units/ml) after the virus had been in activated at 60°C for 30 minutes and stored at -20°C in aliquots of 0.3 ml.

(b) Mitogens

(i) Phytohemagglutinin -P (PHA-P)

75 mg of PHA-P (Difco Laboratories, Detroit, Michigan 48201. U.S.) in 5 mls powder were mixed with 250 mls of normal saline giving a stock concentration of 0.3 mg or 300 ug/ml. It was stored in 10 ml aliqouts at -20°C.

(ii) Pokeweed (PWM)

PWM (Ciba. Geity) had a concentration of 5 mg/ml and was stored at -20°C.

(B) Methods

(1) Lymphocyte Transformation Assay

Appropriate volumes of antigens and mitogens (see Table 4) were added to one milliliter of medium RPMI 1640 (Weymouth;), (6.25 mls of penicillin-streptomycin 10,000 u/ml penicillin; 10,000 mcg/ml Streptomycin per liter) with 30% AB serum. After mixing the suspension thoroughly, 0.1 ml was dispensed into 3 flat-bottomed microtiter culture wells for each antigen and mitogen. The control wells had mediun RPMI 1640 with 30% AB serum without any mitogen or specific antigen excepting controls for HSV-1 and HSV-2 antigens. One-tenth milliliter of lymphocyte suspension in RPMI 1640 at $1x10^6$/ml and $2x10^6$/ml were added to mitogens and antigens respectively, including the control wells. The cultures were maintained at 37°C in a 5% carbon dioxide humidified atmosphere. (Carbon Dioxide Incubator Hotpack-Philadephia Pa.). In the morning of day 6, each well was pulsed with 0.05 ml of RMPI 1640 containing 1 uC of tritiated thymidine ([methyl -^{3}H] thymidine - 1.0 mC/ml (The Radiochemical centre, Amersham, England.) diluted 50x in RPMI 1640;). After 6-8 hours the cells were harvested using a cell harvester (Skatron A.S. type

Lierbyen, Norway) onto glass filters which were air dried individually for at least 16 hours. The dried glass filters were put into scintillation counting vials and 9mls of scintillation fluid were added to each vial (0.01% of dimethyl - POPOP 1-4-bis[2-4 (-methyl-5-Phenylozolyl)] benzene (Scintillation Grade); 0.5% PPO - 2, 5 -diphenyloxazole (Scintilation Grade) in toulene, Amersham/Searle, I11.) The activity in each vial was counted using a liquid scintillation beta-counter (Beckman LS-230 Liquid Scintillation System) and the counts were recorded as counts per minute (CPM) compensated for quenching.

Dose response curves were performed to determine the optimal doses of antigens and mitogens Figures 1-3. Optimal doses are shown in Table 6 some of which had been previously determined.

$$\text{Stimulation Index (SI)} = \frac{\text{CPM Stimulated lymphocytes}}{\text{CPM Unstimulated lymphocytes}}$$

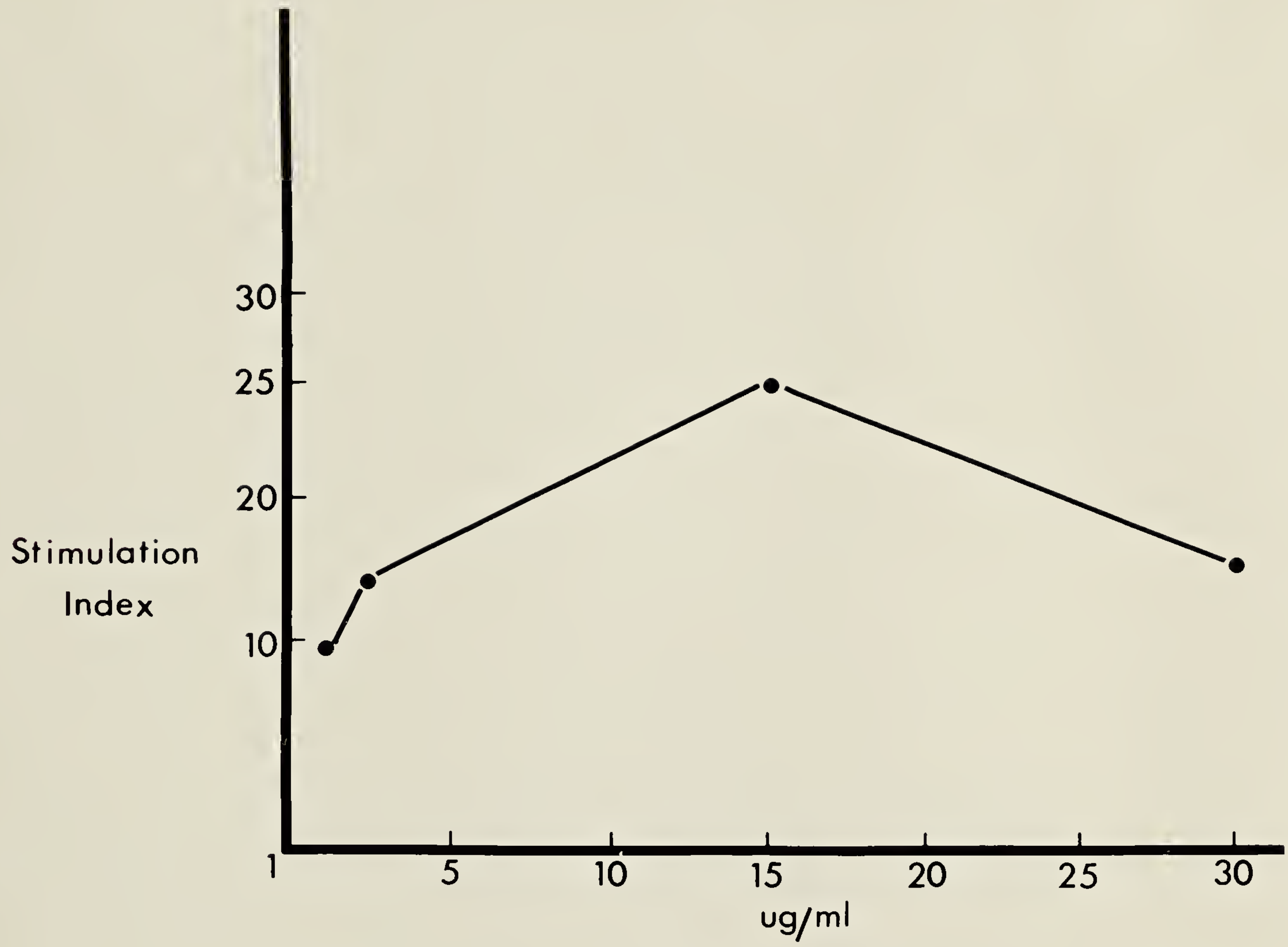

Figure 1. PHA-P Dose response curve

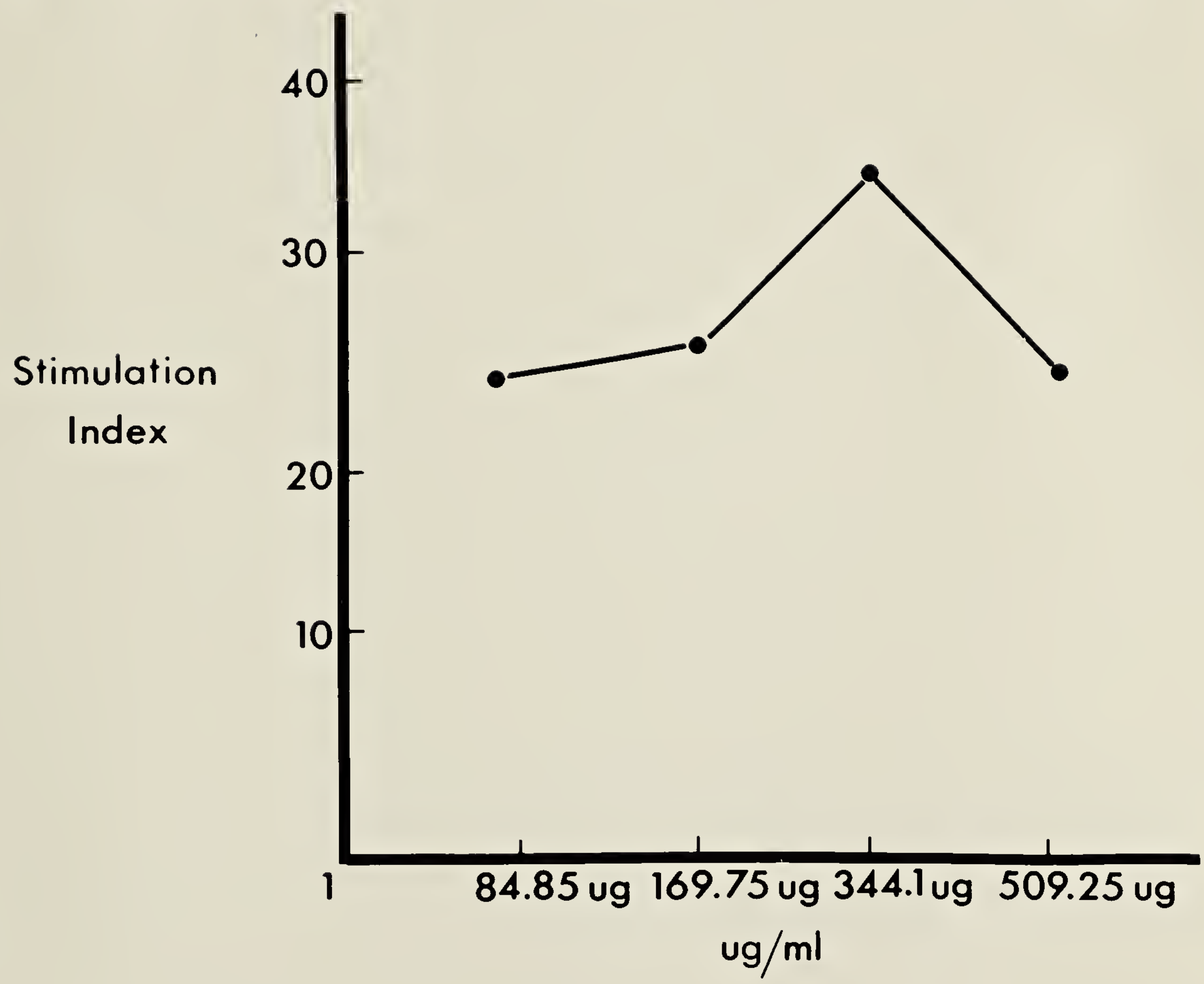

Figure 2. HSV-1 Dose response curve

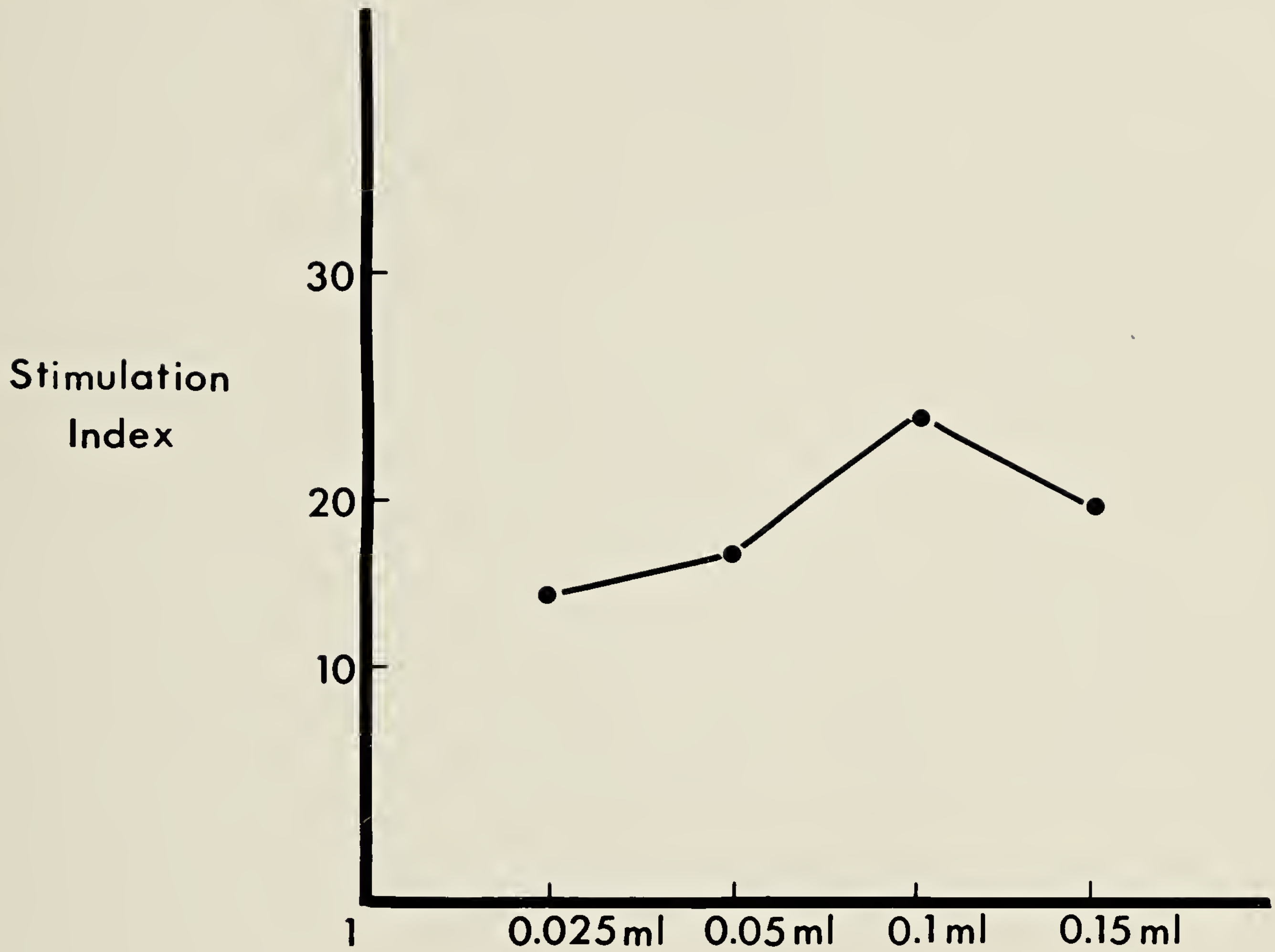

Figure 3. HSV-2 Dose response curve

Table 6

Amounts and concentrations of antigens and mitogens in cell cultures.

Stimulating Agent	Amount per well	Conc.per ml. in well
PHA-P, 0.05 ml.	1.43 ug	7.15 ug
PHA-P, 0.1 ml.	2.73 ug	13.65 ug
PWM, 0.01 ml.	4.95 ug	24.75 ug
Varidase, 0.05 ml.	50 uts	250 uts
Varidase, 0.1 ml.	100 uts	500 uts
Vaccinia virus 0.05 ml.	500 pfu	2500 pfu
Vaccinia virus, 0.1 ml	1000 pfu	5000 pfu
HSV-1, 0.05 ml.	33.95 ug	169.75 ug
HSV-1, 0.1 ml.	68.82 ug	344.1 ug
HSV-2, 0.05 ml.	not determined	not determined
HSV-2, 0.1 ml.	not determined	not determined

(2) Antibody-Dependent Cell-Mediated Cytotoxicity

(a) Macro Assay

After trypsinization the uninfected and infected cells (targets) were washed 4 times in magnesium and calcium-free Hanks' BSS (appendix C (a)). Viability was determined with trypan blue exclusion by counting targets (104) in a hemocytometer and was usually over 95%. The cell concentration was also determined at this time. The targets were labelled with ^{51}Cr (New England Nuclear) using 50 muC/1 x 10^6targets (85). They were then placed in a shaking water-bath at 37°C; and after 1 hour they were removed and washed 4 times in Hanks' BSS with 10% fetal calf serum, and the concentration was adjusted to 2.5 x 10^4/ml. The effector cell suspension was adjusted to 2.5 x 10^6/ml in TC medium 199.

All tests were performed in plastic tubes in duplicate (12 x 75 mm) (Kimble, Division of Owens - Ill.) in which 0.1 ml of test serum; 1 ml of effector cell suspension and 0.025 ml of target cell suspension were mixed. The effector to target cell ratio used was100:1 (104). This reacting mixture was incubated at 37°C in a 5% carbon dioxide humidified atmosphere for 3 hours. The tests for maximum ^{51}Cr release were performed by freezing targets at -20° and thawing 3 times in TC 199. The reactions of all

the tests were stopped by addition of 1 ml of ice-cold Hanks BSS and the suspension was centrifuged at 1200 rpm for 8 minutes at 4°C. The supernatant and cell pellet were counted separately by gamma counter (80000 Gamma Sample Counter Wallac). The counts were recorded as counts per minute (CPM).

Calculations

(a) % Background ^{51}Cr release

$$= \frac{\text{cpm in Supernate with TC199} \times 100}{\text{cpm in Supernate + cells}} = B\%$$

(b) % Test sera ^{51}Cr release

$$= \frac{\text{cpm in Supernate with test serum} \times 100}{\text{cpm in Supernate + cells}} = T\%$$

(c) % Maximum ^{51}Cr release (3 freeze-thaws)

$$= \frac{\text{cpm in Supernate + TC 199} \times 100}{\text{cpm in Supernate + cells}} = M\%$$

(d) % Control ^{51}Cr release

Uninfected target + effector cells
Infected target + effector cells

$$= \frac{\text{cpm supernate} \times 100}{\text{cpm supernate + cells}} = C\%$$

(e) % Corrected ^{51}Cr release

$$= \frac{(T - B) - (C - B) \times 100}{M}$$

$$= \frac{(T - C) \times 100\%}{M} = \text{K-HSV}$$

Experiments which included targets plus serum alone did not show any significant ^{51}Cr release above background nor did targets incubated with effector cells alone.

(b) Micro Assay

A Micro assay technique as described by Aeiljlemaker et al 1975 (128) was adopted. Target and effector cell suspensions were prepared exactly as for 2 (a) (vide supra) except that the final cell concentrations were 5 x 10^4/ml and 5 x 10^6/ml respectively. Round-bottomed microtiter plates (Flow Laboratories, 936 Westthy Pack Bld. Inglewood Ca.) were used. Each well contained a volume of 0.15 ml, consisting of 0.05 ml of each serum, effector cell suspension and target cell suspension. The optimal effector cell : target cell ratio remained at 100:1. To control wells were added targets with Tc199 alone, or targets and effector cells alone. The maximum percentage of ^{51}Cr that could be released was determined by adding a lysing agent, saponin 1% (A product of Coulter Diagnostics, Inc. Miami Springs, Florida 33166). All tests were done in duplicate. To be able to calculate the % ^{51}Cr release, the total CPM per well must be known. Thus a series of counting tubes were filled with 0.05 ml of each target at different stages of filling

plates. The plates were incubated at 37°C in a 5% carbon dioxide humidified atmosphere for 3 hours. The plates were then centrifuged at 1200 rpm in fitting baskets for 8 minutes at 4°C. From each well 0.1 ml of supernatant was transferred into a counting tube using an "Eppendorf" pipette. The radioactivity was determined by using a Wallac Gamma Counter as in 2 (a) (vide Supra).

Calculations:

$$\% \ ^{51}\text{Cr release per well} = \frac{\text{cpm in 0.1 ml supernate x 1.5}}{\text{Total cpm}} \times 100$$

<u>NB</u>. Total volume per well is 0.15 ml.

<u>and</u> only 0.1 ml is actually counted, otherwise the remaining calculations are as in 2(a).

IV RESULTS

(A) Statisitcal Analysis of Data.

The results of lymphocyte transformation tests and serological activity in ADCMC were expressed as HSV-1/HSV-2 and K-HSV-1/K-HSV-2 ratios respectively. The raw data and logarithms of transformation indices of mitogens PHA and PWM; and antigens varidase and vaccinia were also used (129). Statistical significance was determined by use of student-t-test, X^2, analysis of variance by multiple range student Neuman-Keuls procedure as well as correlation coefficient (130). Results were considered to be signigicant if the p value was 0.05 or less.

(B) Lymphocyte Transformation Results

(1) Optimal Doses of Lymphocyte Stimulating Agents

Initial experiments were performed to determine the optimal doses of mitogens and antigens. With the exception of PWM, 2 doses of each mitogen or antigen were used in each subject tested. The optimal doses obtained and used in all the experiments are shown Figures 1-3 and Table 6.

(2) Results of HSV-1/HSV-2 ratios

On the basis of previously published studies, see Chap. II(c)(3) when a ratio of less than 1 was obtained,

the subject was deemed to be responding to HSV-2 antigen. The following patients responded to HSV-2 antigen: 6 of 8 patients with untreated carcinoma-in-situ; 2 of 4 patients with treated carcinoma-in-situ; 9 of 12 patients with squamous cell carcinoma of the cervix stages 1-3; 1 of 2 patients with adenocarcinoma of the cervix; 3 of 11 patients with carcinoma of the uterine body and or ovaries; 3 of 5 patients with lung cancer; 6 of 8 healthy individuals; and 8 of 18 patients with medical illnessess. The value of X^2 for the number of patients with ratios of less than 1 among patients with squamous cell carcinome of the cervix and carcinoma of the uterine body and ovaries was significant at the p 0.025 level (Table 7). The means of the ratios are shown Table 8. A student-t-test between the means of ratios in patients with untreated carcinoma-in-situ patients with carcinoma of uterine body and ovaries was statistically significant, $p < 0.025$; and the difference between the means of all patients with carcinoma-in-situ and carcinoma of the uterine body and or ovaries was significant, $p < 0.05$; and the difference between the means of the patients with squamous cell carcinoma stages 1-3 and carcinoma of the uterine body and or ovaries was also statistically significant, $p < 0.05$ (Table 7 and 8). The stimulation indices of the groups of subjects studied are shown in Figures 4-6.

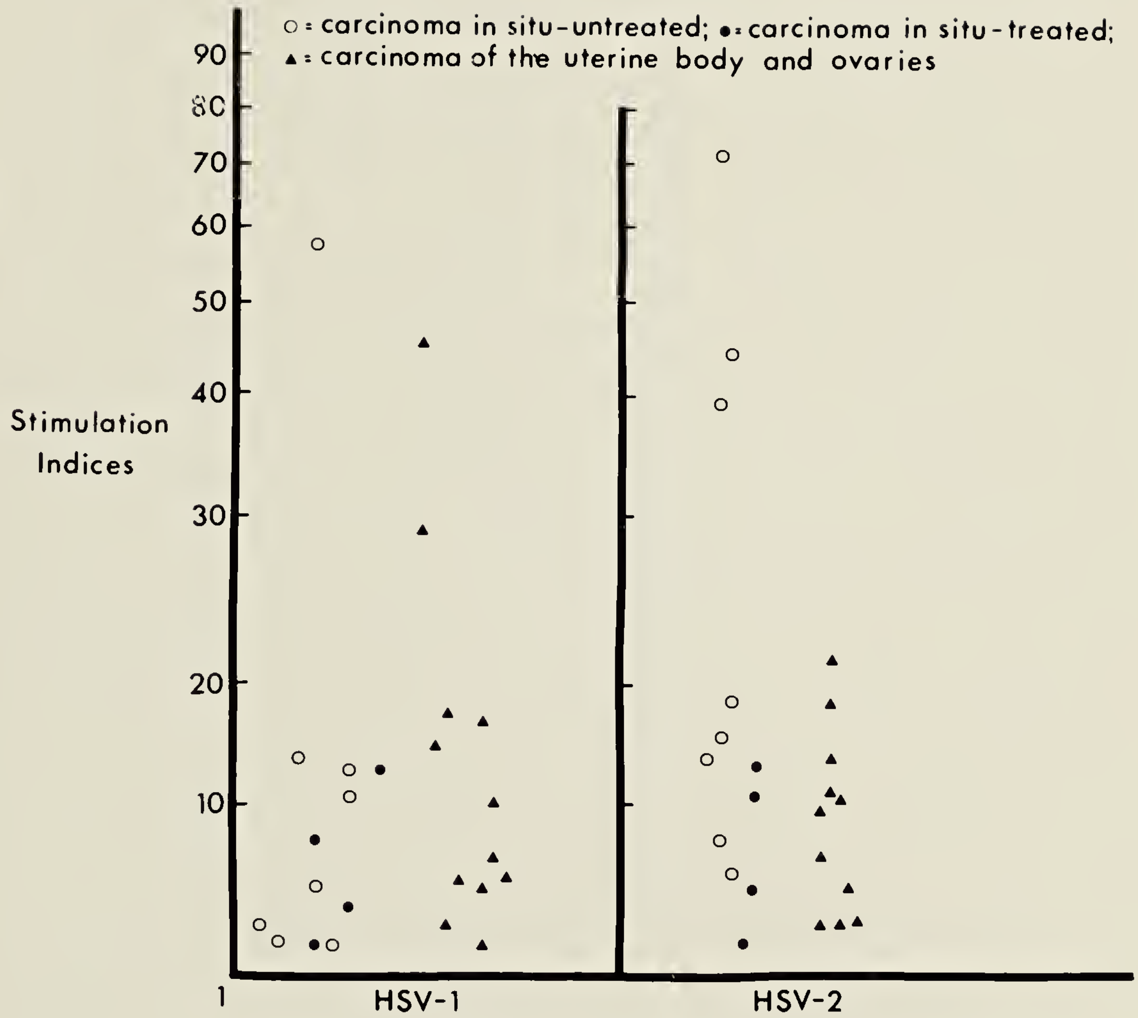

Figure 4. Stimulation indices in subjects with untreated carcinoma in situ, treated carcinoma in situ and carcinoma of the uterine body and ovaries

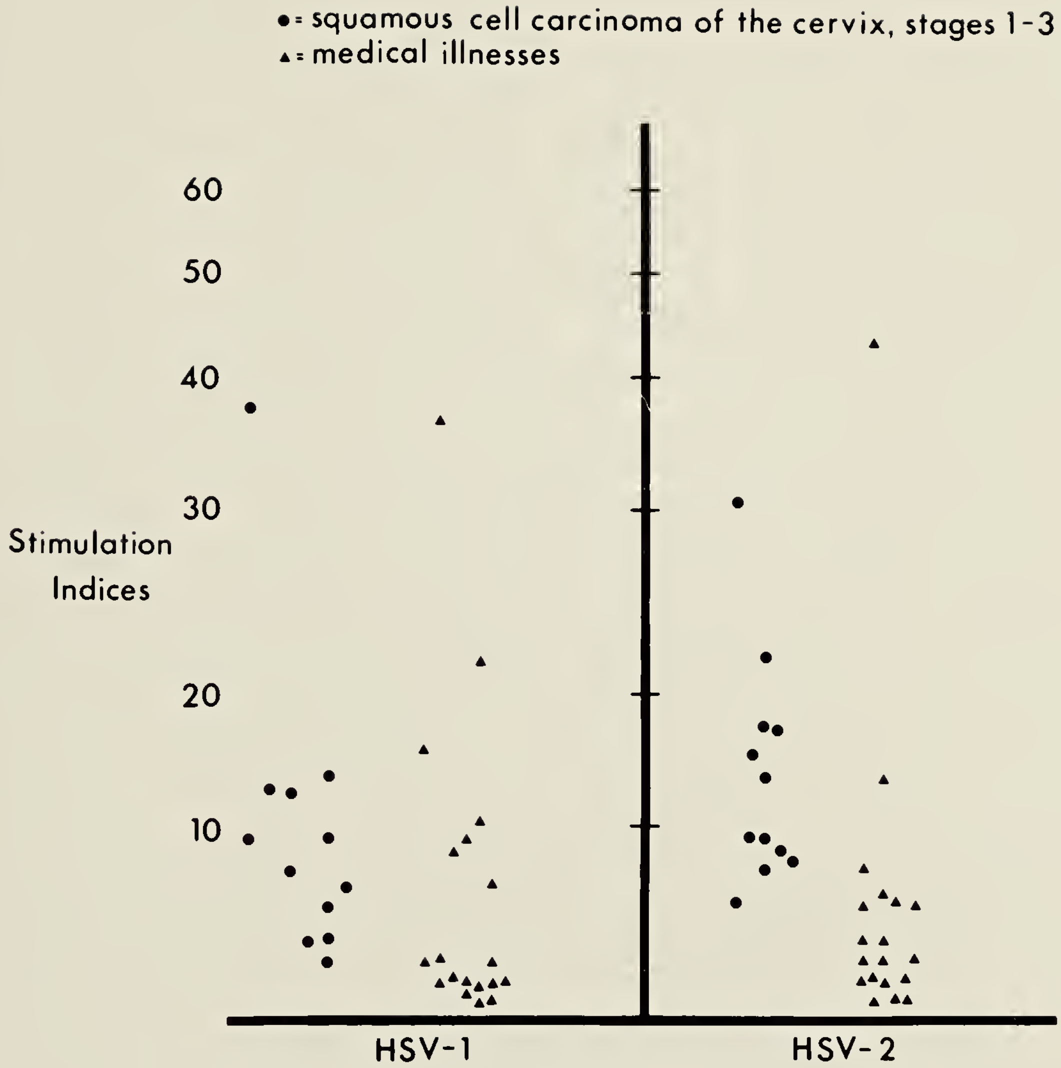

Figure 5. Stimulation Indices of subjects with squamous cell carcinoma of the cervix, stages 1-3 and medical illnesses

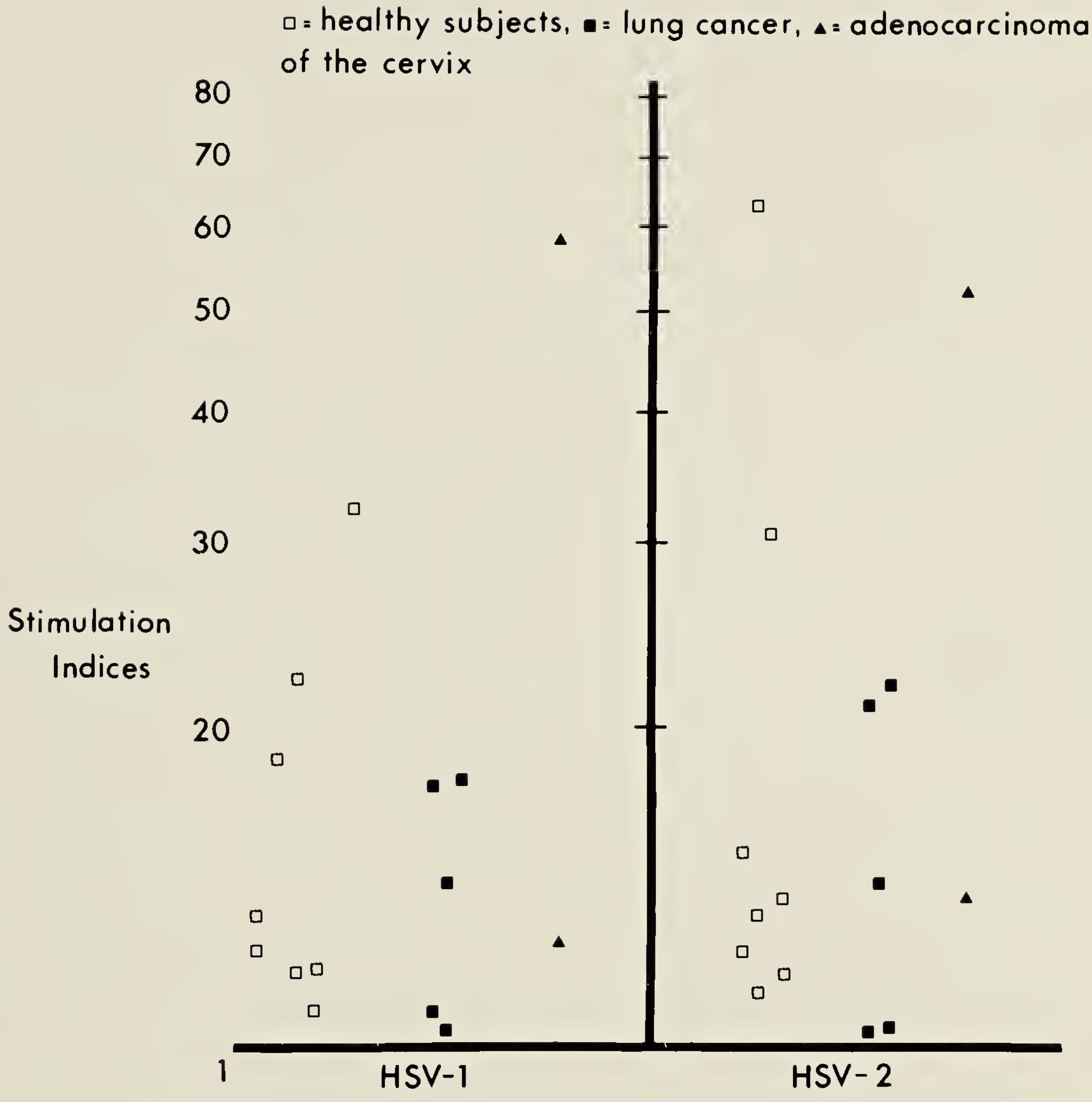

Figure 6. Stimulation indices of healthy subjects, with lung cancer and adenocarcinoma of the cervix

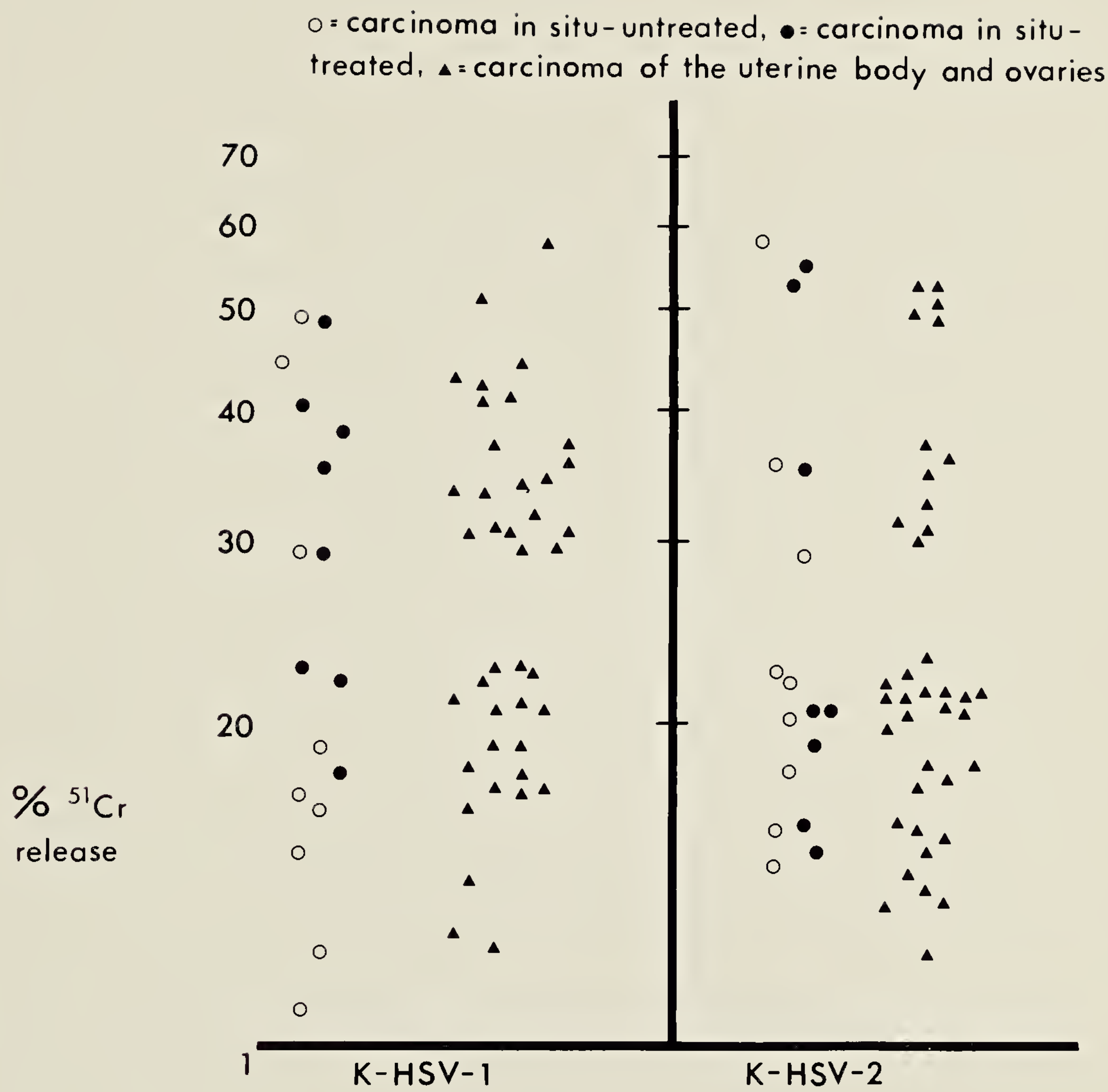

Figure 7. % ^{51}Cr release in subjects with untreated carcinoma in situ, treated carcinoma in situ and carcinoma of the uterine body and ovaries

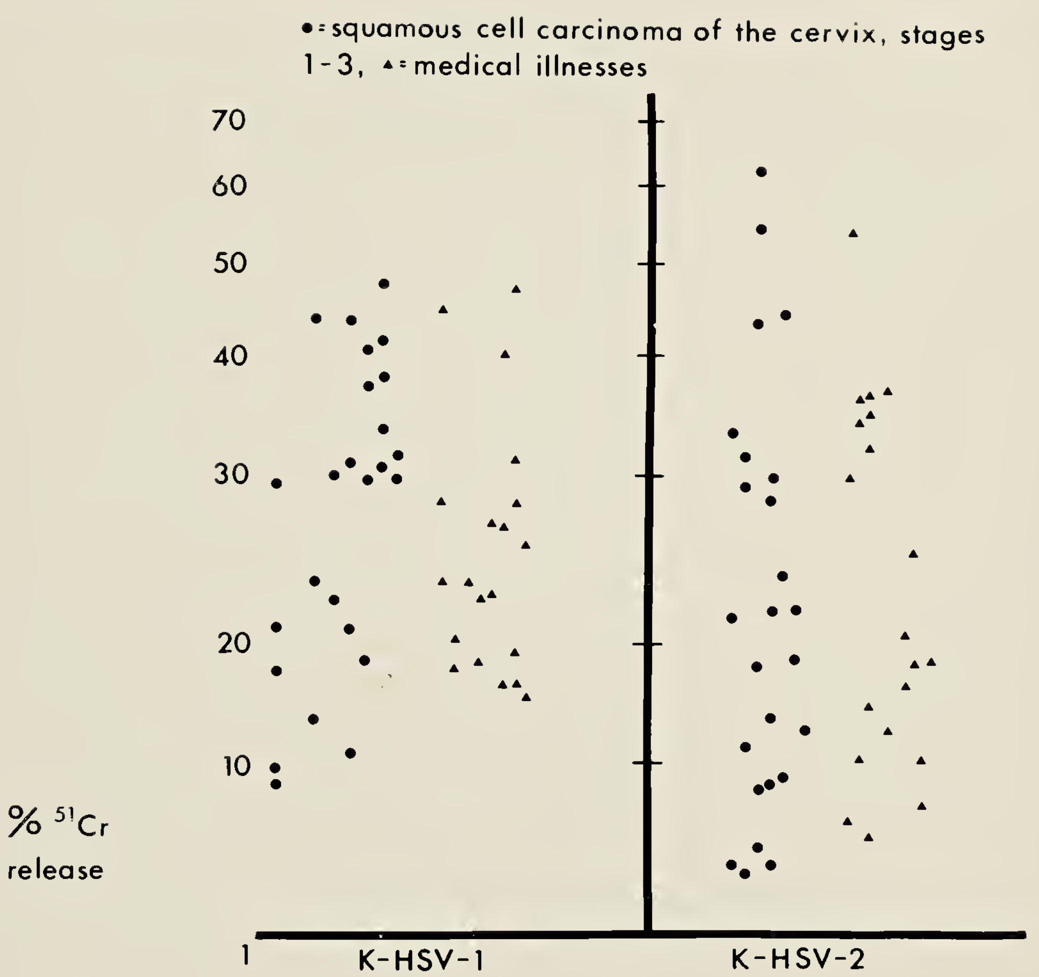

Figure 8. % ^{51}Cr release in subjects with squamous cell carcinoma of the cervix, stages 1-3 and medical illnesses

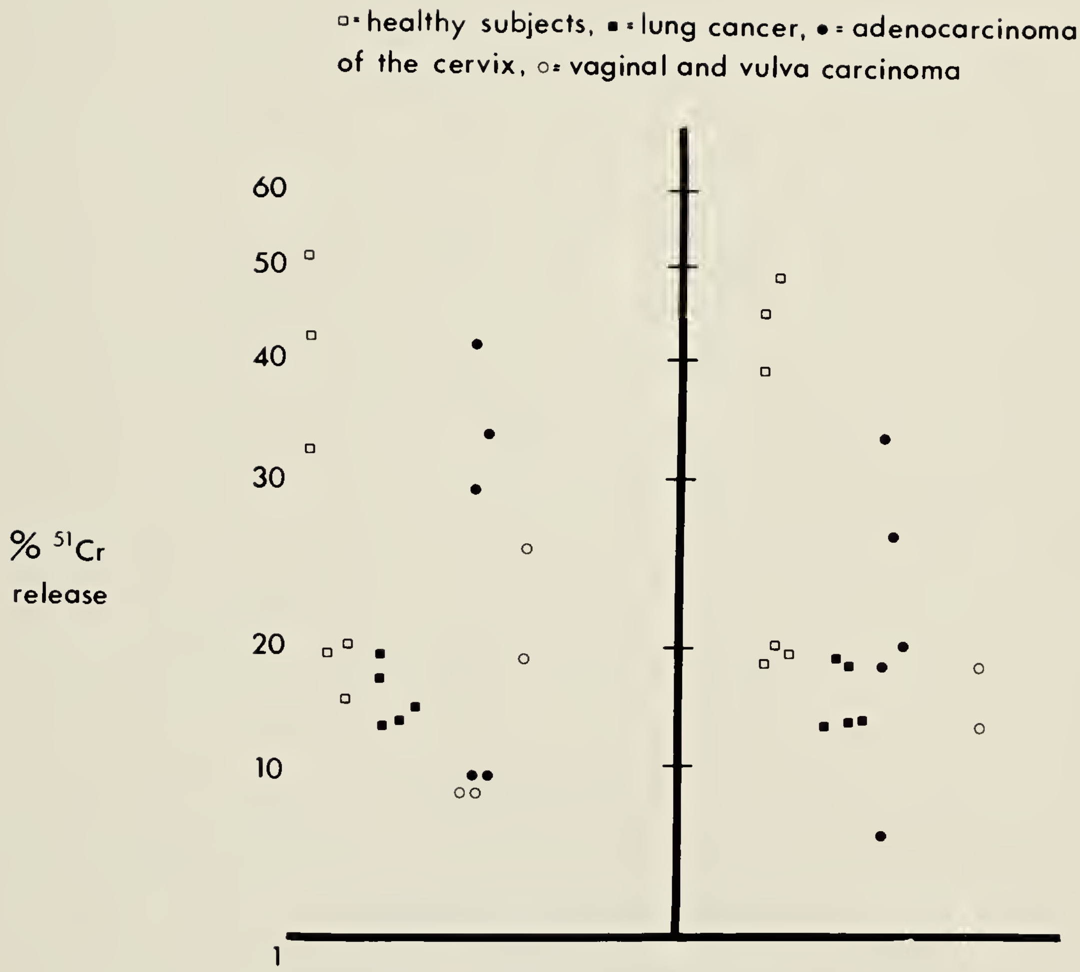

Figure 9. % ^{51}Cr release in healthy subjects, lung cancer, adenocarcinoma of the cervix and vaginal and vulva carcinoma

Table 7

Number of subjects tested with a lymphocyte* transformation (TI) of <1 and also with a serological ADCMC ratio of <1

Diagnosis	number tested	$\frac{\text{SI-HSV-1}}{\text{SI-HSV-2}} < 1$	number tested	$\frac{\text{K-HSV-1}}{\text{K-HSV-2}} < 1$
Carcinoma-in-situ -Untreated	8	6	9	7
Carcinoma-in-situ -treated	4	2	8	3
Squamous cell carcinoma of cervix Stages 1-3 -treated	12	9 (a)	25	7 (a)
Adenocarcinoma of cervix	2	1	5	1
Carcinoma of uterine body and or ovaries	11	3 (a)	40	14 (a)
Vaginal and vulva carcinoma	ND	ND	2	0
Lung cancer	5	3	5	2
Healthy individuals	8	6	6	2
Medical illnesses	18	8	20	8

$$\text{*Transformation Index} = \frac{\text{SI-HSV-1}}{\text{SI-HSV-2}} = \text{TI}$$

$$\frac{\text{K-HSV-1}}{\text{K-HSV-2}} = \frac{\text{\% Corrected } ^{51}\text{Cr release from HSV-1 targets}}{\text{\% Corrected } ^{51}\text{Cr release from HSV-2 targets}}$$

a: X^2 of number of patients with squamous cell carcinoma with ratios <1 with carcinoma of uterine body and or ovaries, $p < 0.025$ (with Yate's correction)

Table 8

Means of lymphocyte transformation indices and serological ADCMC ratio

Diagnosis	number tested	SI-HSV-1 / SI-HSV-2 mean±SD	number tested	K-HSV-1 / K-HSV-2 mean±SD
Carcinoma-in-situ -Untreated	8	0.7±0.4 (a)	9	0.8±0.4 (c)
Carcinoma in situ treated	4	1.0±0.6	8	1.4±0.8
Squamous cell carcinoma of cervix stages 1-3.	12	0.8±0.4 (b)	25	1.4±0.8
Adenocarcinoma of cervix	2	0.9±0.3	5	1.6±0.8
Carcinoma of uterine body and or ovaries	11	1.7±1.3 (a) (b)	40	1.3±0.7 (c)
Vaginal and vulva carcinoma	ND	ND	2	1.4
Lung cancer	5	1.4±1.4	5	1.0±0.2
Healthy individuals	8	1.0±0.9	6	1.1±0.5
Medical illness	18	2.0±4.3	20	1.6±1.0

(a) t-test-means of patients with untreated carcinoma-in-situ against means of patients with carcinoma of the body of the uterus and or ovaries $p < 0.025$

(b) t-test-means of patients with squamous cell carcinoma stages 1-3 against means of patients with carcinoma of thebody and or ovaries. $p < 0.05$

(c) t-test-means of patients with untreated carcinoma-in-situ against means of patients with carcinoma of the uterine body and or ovaries $p < 0.05$

(3) Stimulation Indices by PHA, PWM Mitogen; and Varidase and Small pox antigens

There was no difference in healthy and patients studied when mitogens PHA and PWM; and antigens varidase and vaccinia were used in lymphocyte transformation tests.

(C) Serological Activity in ADCMC

(1) Optimal Effector: Target cell Ratio:

The optimal effector: target cell ratio had been determined previously and had been found to be between 75:1 and 100:1 (104) for HSV-1 targets and the same ratio was used for HSV-2 targets.

(2) Results of ADCMC

The subjects were considered to have antibodies to HSV-2 when the activity of HSV-1/HSV-2 was less than 1. Antibodies to HSV-2 were found in 7 Of 9 patients with untreated carcinoma-in-situ; 3 of 8 patients with treated carcinoma-in-situ; 7 of 25 patients with treated squamous cell carcinoma of the cervix, 1 of 5 patients with adenocarcinoma of the cervix; none among 2 patients with vaginal and vulva carcinoma; 14 of 40 patients with carcinoma of uterine body and or ovaries; 2 of 5 patients with lung cancer; 2 of 6 healthy individuals and 8 of 20 patients with medical illnesses. (Table 7). The difference of the means of ratios in patients with carcinoma-in-situ and carcinoma of the uterine body and or ovaries was

statistically significant, $p < 0.05$. The % ^{51}Cr release for the groups of subjects tested are shown in Figures 7-9.

(D) Lymphocyte Transformation tests and ADCMC Considered together.

This was done to find out how many of all the patients who had HSV-1/HSV-2 <1 also had K-HSV-1/K-HSV-2 <1 and vice versa. Patients found to have K-HSV-1/K-HSV-1 <1 when they had HSV-1/HSV-2 <1 were: 4 of 6 with Carcinoma in situ; 4 of 9 with squamous cell carcinoma of cervix stages 1-3; 1 of 3 with carcinoma of the uterine body and or ovaries; 2 of 3 with lung cancer, 2 of 6 healthy individuals and 3 of 8 with medical illnesses. The patients found to have HSV-1/HSV-2 <1 when they had K-HSV-1/K-HSV-2 <1 were: 4 of 7 with untreated carcinoma-in-situ; 7 of 7 with squamous cell carcinoma of cervix stages 1-3; 1 of 11 with carcinoma of the uterine body and or ovaries, 1 of 2 with lung cancer; 1 of 2 healthy individuals and 2 of 8 with medical illnesses. All the patients with squamous cell carcinoma of the cervix stages 1-3 who had evidence of humoral immunity also had evidence of CMI. (Tables 9 and 10).

Table 9

Number of Subjects with $\frac{\text{K-HSV-1}}{\text{K-HSV-2}} < 1$ when $\frac{\text{HSV-1}}{\text{HSV-2}} < 1$

	No. with $\frac{\text{HSV-1}}{\text{HSV-2}} < 1$	No. with $\frac{\text{K-HSV-1}}{\text{K-HSV-2}} < 1$
Carcinoma in situ untreated	6	4
Squamous Cell Carcinoma Stages 1-3	9	4
Carcinoma of the uterine body and or ovaries	3	1
Lung Cancer	3	2
Healthy individuals	6	2
Medical Illness	8	3

Table 10

Number of Subjects with $\frac{HSV\text{-}1}{HSV\text{-}2} < 1$ when $\frac{K\text{-}HSV\text{-}1}{K\text{-}HSV\text{-}2} < 1$

	No. with $\frac{K\text{-}HSV\text{-}1}{K\text{-}HSV\text{-}2} < 1$	No. with $\frac{HSV\text{-}1}{HSV\text{-}2} < 1$
Carcinoma in situ untreated	7	4
Squamous Cell Carcinoma Stages 1-3	7	7
Carcinoma of the uterine body and or ovaries	11	1
Lung Cancer	2	1
Healthy individuals	2	1
Medical Illness	8	2

(E) Repeated Tests:

It was possible to follow only 4 patients who were first studied when they were having cone biopsy and a diagnosis of carcinoma-in-situ documented. These patients were studied again 2-3 months after therapy by conization. The mean lymphocyte transformation index remained typical of response to HSV-2 antigen. However, the mean K-HSV-1/K-HSV-2 had risen and with a wider standard deviation. The paired t-test showed that the first and the second means of the tests were not significantly different (Table 11) from each other.

Table 11

Results in patients tests before and after therapy

	Number Tested	Mean HSV-1 / HSV-2	Number Tested	Mean K-HSV-1 / K-HSV-1	p Value
*1st test	3	0.4±0.4	4	0.9±0.1	NS
2nd test	3	0.7±0.3	4	2.1±1.5	

* 1st test - before treatment.

2nd test - repeat test - after treatment.

NS - Not Significant.

(F) Groups of Patients treated by Radiotherapy and Hysterechomy

Nearly all patients with invasive cervical carcinoma were treated by radiotherapy, 55% (22) with uterine carcinoma and 12% (2) with carcinoma-in-situ (Table 12).

82% (33) of the patients with uterine carcinoma were treated by total hysterectomy, 41% (7) with cervical carcinoma stage 1 and 10% (1) with cervical carcinoma stage 2. There was only one patient with cervical carcinoma stage 3 and she was treated by both radiotherapy and hysterectomy (Table 12 and 13).

There was no statistical difference between the results of the tests in the group of patients who had radiotherapy compared to those who had surgery.

(G) Distribution of Parity

In distribution of parity (Table 14) there was one patient with cervical carcinoma stage 1 had parity of 19, and this raised the mean parity for the entire group and certainly widened the standard deviation. The mean parity for the other subgroups was 3.

There was no significant correlation between the tests performed and parity.

Table 12

Number of Patients treated by radiotherapy

Diagnosis	%	Number
Carcinoma-in-situ	12	(2)
Cervical Carcinoma Stage 1	100	(14)
Cervical Carcinoma Stage 2	90	(9)
Cervical Carcinoma Stage 3 (1patient)	100	(1)
Uterine Carcinoma	55	(22)

Table 13

Number of Patients treated by hysterectomy

Diagnosis	%	Number
Carcinoma-in-situ	41	(7)
Cervical Carcinoma Stage 1	14	(2)
Cervical Carcinoma Stage 2	10	(1)
Cervical Carcinoma Stage 3 (1patient)	100	(1)
Uterine Carcinoma	82	(33)

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Table 14

Distribution of Parity

	Range	Mean ± S.D.
Carcinoma-in-situ	0-6	3 ± 2
Cervical Carcinoma Stages 1-3	0-19	5 ± 4
Carcinoma of the uterine body and or ovaries	0-8	3 ± 2

(H) Duration of Disease from Time of Diagnosis

The duration of the disease in some patients with carinoma in situ was recorded as zero (0) because these patients were studied at the time the diagnosis was made (Table 15), and 2 patients have been followed for 21 years. The patient with cervical carcinoma stage 3 when tested had been followed for 6 months (0.5 year).

There was a significant correlation between the duration of the disease and the ratio K-HSV-1/K-HSV-2 but not with ratio HSV-1/HSV-2 which is the transformation index (Table 16).

Table 15

Duration of disease from time of diagnosis

Diagnosis	Range	Mean ± S.D.	Number
Carcinoma-in-situ	0-10	6.0 ± 3.5 years	(17)
Cervical Carcinoma Stage 1	0.8-21	7.2 ± 6.3 years	(14)
Cervical Carcinoma Stage 2	1-17	7.2 ± 5.6 years	(10)
Cervical Carcinoma Stage 3	0.5	0.5 year	(1)
Carcinoma of the uterine body and or Ovaries	0.5-21	6.0 ± 5.4 years	(40)

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[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 16

Correlation Between Duration of Disease from Time of Diagnosis and Tests

Ratio	Number tested	Corr.Coef.*	P Value
$\frac{HSV\text{-}1}{HSV\text{-}2}$	24	0.16	NS
Log $\frac{HSV\text{-}1}{HSV\text{-}2}$	24	0.16	NS
$\frac{K\text{-}HSV\text{-}1}{K\text{-}HSV\text{-}2}$	85	0.24	<0.02
Log $\frac{K\text{-}HSV\text{-}1}{K\text{-}HSV\text{-}2}$	85	0.27	0.01

*Correlation Coeffient

NS = Not Significant

V DISCUSSION

It has been reported that all subjects susceptible to circumoral herpes have CMI against virus directed antigens expressed on the surface in ^{51}Cr assays. The same patients, where tested, all had positive skin tests to herpes antigen and their lymphocytes were stimulated by in vitro incubation with the same antigen. The results with ^{51}Cr release agree very closely with the results of lymphocyte proliferation and skin tests. (102, 104)

In the present study evidence is presented that patients with Carcinoma-in-situ and squamous cell carcinoma of the cervix are indeed able to mount specific cell mediated immune responses to the virus, as detected by HSV-2 antigen-stimulated lymphocyte proliferation; and further that untreated patients show a corresponding high frequency of humoral immunity as detected by antibody dependent cell-mediated cytotoxicity using ^{51}Cr release assay (ADCMC). (Table 7). This demonstrates that a high percentage of patients with carcinoma-in-situ and squamous cell caracinoma of the cervix have previously been infected by herpesvirus type 2. When these patients have been treated successfully, the level of antibodies declines and the number of patients with detectable humoral immunity drops. A study of patients before therapy and after therapy showed that the frequency of anti-HSV-2 antibody response fell following therapy while lymphocyte transformation tests still showed specificity to HSV-2 antigen (Table 11). Further the humoral immunity as assessed by ADCMC

in all the treated group of patients was considerably lower, 3 of 8 patients with treated carcinoma-in-situ and 7 of 25 patients with squamous cell carcinoma of the cervix stages 1-3 as compared to 7 of 9 patients with untreated carcinoma-in-situ.

There was a very significant correlation between duration and disease from time of diagnosis in years and ADCM serological activity while no such correlation was demonstrable with CMI tests (Table 16). It therefore seems that HSV-2 antibody tend to fall with therapy and time, while CMI remains unchanged at least for the duration of disease studied. Other studies have shown that specific neutralizing antibodies to HSV-2 in patients with cervical carcinoma drop after therapy (124). This suggests that when assessing immunity in patients with carcinoma-in-situ or squamous cell carcinoma of the cervix, both cell-mediated and humoral immunity should be assessed at the same time. While the demonstration of CMI may indicate prior HSV-2 infection, anti-HSV-2 sera reactivity may be an indicator of the presence of a lesion containing viral structural proteins in patients with carcinoma-in-situ or squamous cell carcinoma.

Our control group of 40 patients with carcinoma of the uterine body and or ovaries had a much lower incidence of CMI and sera reactivity in ADCMC in respect to HSV-2, and was shown in 3 of 11 patients and 14 of 40 patients respectively. The results in these patients as a group were significantly different from either the group of patients with carcinoma-in-

situ or squamous cell carcinoma of the cervix stages 1-3 (Table 7).

3 of 5 and 2 of 5 patients with lung cancer had evidence of CMI and humoral immunity to HSV-2 respectively. The means of the ratios of HSV-1/HSV-2 and K-HSV-1/K-HSV-2 did not differ significantly from the means of the corresponding ratios in patients with carcinoma-in-situ and squamous cell carcinoma. The role of herpes in lung cancer is far from clear, and adequate studies have not been done to determine if there is any cause and effect relationship. There have, however, been indirect studies. Smith et al (131) produced evidence for an association between herpes simplex virus, heavy cigarette smoking and head and neck squamous cell carcinoma, in that these subjects had significantly raised anti-HSV antibodies of IgA type. The titers dropped after an adequate period of successful treatment in those who had squamous carcinoma and had become tumor free. Their technique did not try to distinguish between antibodies directed primarily at HSV-1 and HSV-2. Further Mandel et al (132) have shown that there is an elevation of local production of salivary IgA as well as serum IgA in patients with oropharyngeal and bronchopulmonary carcinoma. More studies will have to be done on patients with lung cancer, and preferably bearing in mind the differences between HSV-1 and HSV-2 before conclusions are drawn.

Among the healthy individuals, all females, 6 of 8 had evidence of CMI and 2 of 6 had humoral immunity to HSV-2.

These individuals were unselected and tested at random. This data suggests that even among healthy females, the frequency of individuals already sensitized to HSV-2 can be alarmingly high. Previous studies where controls have been included have paid more attention to evidence of neutralizing antibodies and there have been few studies which have tested CMI and humoral immunity at the same time. One of Kessler's studies (133) had shown that 58.6% of a group of nuns had neutralizing HSV-2 antibodies. It has been suggested that such individuals especially those with high titers should be followed, to detect early causes (134).

In this study 1 of 2 and 1 of 5 patients with adenocarcinoma had evidence of CMI and humoral immunity to HSV-2. The number of patients studied is small but it would appear that the frequency of antibodies in these patients may be low. Two patients had vaginal and vulva carcinoma and these were not tested for CMI; but they were tested for humoral immunity and both had no antibodies to HSV-2. Adenocarcinoma of the cervix has not been associated with HSV-2, and the number of the patients with vaginal and vulva carcinoma is small so that no conclusions can be drawn.

4 of 7 patients with carcinoma-in-situ who had HSV-2 antibodies also had evidence of CMI, and 7 of 7 patients with squamous cell carcinoma of cervix stages 1-3 who had HSV-2 antibodies also had evidence of CMI. The corresponding number of patients with carcinoma of the uterine body and or ovaries and medical illnesses respectively were 1 of 11 and 2 of 8.

However of those patients with carcinoma-in-situ and squamous cell carcinoma of the cervix who had evidence of CMI to HSV-2, a smaller proportion had this antibody specificity. (Tables 9 and 10) In patients with lung cancer 2 of 3 with CMI to HSV-2 had HSV-2 antibodies. This is further evidence to support the fluctuating pattern of antibodies to HSV-2 in patients who are treated where evidence of CMI persists while antibodies decline.

There was no difference between healthy and patients when PHA, PWM, varidase and vaccinia were used in lymphocyte transformation tests. Balakrishan and Hanjan in 1975 (135) found that cellular immune responsiveness in patients with carcinoma of the cervix to PHA was significantly lower than that of controls in both AB and autologous serum.

The means of all the indices and ratios of the patients who were treated by radiotherapy were compared to those who had surgery. It was found that there was no statistical difference between the two groups. The mean duration of disease from time of diagnosis in the patients studied was 6-7 years (Table 12 and 15). Yamagata and Green in 1976 (136) studied radiation induced immune changes in patients with cervical carcinoma by using PHA and found that there was a depressed lymphocyte reactivity for at least 5 years following irradiation but lymphocyte reactivity was present in patients who had responded well to radiotherapy with the exception of those with recurrences.

Hysterectomy was performed in 7 of 17 patients with documented carcinoma-in-situ (Table 13). This type of surgery is now very rarely done for this kind of lesion; and among the 17 patients studied only 1 has had hysterectomy becuase of carcinoma-in-situ, over the last 4 years. Cone biopsy procedure is now regarded as both of diagnostic and therapeutic value. Clinical trials are currently under way to evaluate cryosurgery, a technique that has so far given promising results (137).

Rotkin as early as 1964 demonstrated that sexual intercourse before the age of 17 and the history of multiple sexual partners are among the most discriminating variables distinguishing groups of cervical cancer patients from the control groups (138). The continuing study by Kessler has shown that the risk of developing cervical cancer is increased among the second wives of men who previously were married to women who had developed cervical cancer (133). There is however no evidence that penile malignancies are increased in males with herpesvirus infections so that the male may only spread the transmissible agent. It would appear then that whatever methods are applied to detect HSV-2 carriers or subjects infected with HSV-2, the men should not be put aside.

While there are now effective techniques for the treatment of carcinoma-in-situ and squamons cell carcinoma of the cervix, there has been very little accomplished in treating herpesvirus infection. It is too simple to suggest that good hygiene is adequate because it is only part of the

answer. Small pox vaccination has been tried but is ineffective and photoinactivation has been used with variable results and there are theoretical possibilities of producing defective oncogenic viruses by such treatment. Anderson and his coworkers (139) reported in 1973 that when intradermal injection of Bacillus Calmette Guerin (BCG) was given to individuals with chronic vaginal or penile infections due to documented HSV-2 and who had negative skin tests with Purified Protein Derivative (PPD), there was a greater than 50% reduction in recurrences 10 patients. But this study was not controlled in any way.

In Chapter II (C)(2) evidence was presented to indicate the role of immunity in herpesvirus infection; and in the same chapter (C)(5) it became apparent that cervical malignant lesions actually occur in the presence of both cellular and humoral immunity to HSV-2. Further, there was specific immunity present against the HSV antigen designated AG-4 in those with Carcinoma of the Cervix in situ or squamous cell carcinoma. If CMI and humoral immunity are going to be of any therapeutic value, it is obvious that they must be boosted because the natural immunity to HSV-2 is not sufficient to prevent the development of Carcinoma in the affected persons. This however does assume that HSV-2 is an etiologically important agent in Carcinoma-in-situ and squamous cell carcinoma. It is important to clarify this further and to continue to assess other possible factors or cofactors that may be responsible. Retrospective studies of the HSV/ Cervical

Carcinoma association can only provide circumstantial evidence but prospective studies will be necessary to resolve the mystery of this association. Conclusions on the results of subjects with adenocarcinoma of the cervix, vaginal and vulva carcinoma, lung cancer and healthy individuals cannot be drawn because of the small numbers of subjects studied in each of these groups.

BCG and Corynebacterium parvum are possibilities to be applied in an attempt to boost both CMI and humoral immunity in patients with HSV-2. At the present time it does not seem that these same adjuvant immunotherapies need be applied for carcinoma-in-situ or squamous cell carcinoma because of the already existing effective modalities of therapy. The real crux of the problem is to control herpesvirus type 2 in all carriers including the men. The ideal mode of controlling herpesvirus type 2 infection would be to prepare a potent HSV-2 vaccine. This cannot be achieved until the complexity of HSV-1 and HSV-2 cross-reactivity is clarified. When the different HSV-antigenic components are isolated, purified and tested in isolation of each other, then, the rest will not necessarily be easier but will be less complex.

Appendix A

Preparation of HSV-2 Antigen

1. HAE 70 cells with passage number between 15 and 20 were used.
2. The cells were cultured for 24 hrs at 37°C in a 5% carbon dioxide humidified atmosphere and formed confluent monolayers.
3. The HAE cells were then infected with HSV-2 (see appendix B) and left for 72 hours, at which time all cells showed typical cytopathic effect. The uninfected cells provided material for preparing control antigen.
4. The monolayer cells and the supernatant were frozen and thawed 3 times. The cells were vigorously suspended and removed with a pasteur pipette.
5. Virus was inactivated by exposure to ultra violent light.
6. The virus was sonicated for 2 minutes.
7. The antigen was filtered through 0.22 u filters.

Appendix B

Method of Infecting Target Cells

1. HSV-1 and HSV-2 cultured from patients with active lesions were grown in tissue culture and passaged in a continuous line of human amnion Edmonton 70 (HAE 70). The stock HSV-1 and HSV-2 were titrated and frozen in aliquots at -70°C.
2. 3 Roux bottles were seeded with HAE[6] cells in minimal essential medium, (MEM) (6.25 mls of Antibiotic-antimycotic mixture penicillin 10,000 u/ml - fungizone 25 mcg/m. Streptomycin 10,000 mcg/ml per liter) (Gibco) with 2% fetal serum and cultured at 37°C in a 5% carbon dioxide humidified atmosphere.
3. After 24 hours a confluent monolayer of cells was formed and excess growth medium was decanted. Two bottles were each infected with (4×10^5) or 0.5 mls of stock HSV-1 or HSV-2 which had just been thawed at room temperature. The virus are heat labile and were put in cell culture immediately.
4. The bottles were incubated at 37°C and gently rocked after every 10 minutes for 30 minutes.
5. 30 mls of MEM with 2% fetal calf serum were added to the infected cell line and cultured for a further 48 hours for infectivity of cells to occur at 37°C in a 5% carbon dioxide humidified atmosphere.

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1. [illegible]

2. [illegible]

3. [illegible]

4. [illegible]

Appendix C

Trypsinization

(a) Infected HAE and non-Infected HAE cells

1. After 48 hours there was good infectivity of the HAE cells and the non-infected cells (control targets) showed a confluent monolayer when observed under an inverted light, microscope.
2. The growth medium (MEM) was decanted.
3. 0.25% trypsin (Grand Island, Biological Co. N.Y. 14072, U.S.) solution already warmed to 37°C in a water-bath was added in an amount just sufficient to cover the bottom of the cell culture bottle. The bottle was gently rocked for 1 minute and trypsin was then poured off.
4. A further volume of the same concentration of trypsin was added and incubated at 37°C for 3-5 minutes during which time the cells were dislodged from the surface of the bottle.
5. The dislodged cells were pipetted into a centrifuge tube containing Hanks' balanced salt solution (Hanks' BSS) (Microbiological Associates, Bethesda, Maryland). Magnesium and Calcium-free Hanks' BSS was used because it inhibits cell to cell adherence. The cells were monodispersed by thorough pipetting.
6. The cells were spun at 1200 rpm for 7 minutes at room temperature and then washed in Hanks' BSS 3 times after which the cells were ready for counting under hemocytometer.

(b) Maintainance of HAE cells

1. MEM with 10% fetal calf serum was used to grow HAE cells after trypsinsation.
2. When the confluent monolayer was almost formed, the medium was removed and replaced MEM with 2% or 1% fetal calf serum and culture maintained at 37°C in a 5% carbon dioxide humidified atmosphere.

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